IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: DANILO PORRO MICHAEL SAUER Serial No.: 10/606,302 Filed: June 25, 2003 For: ASCORBIC ACID PRODUCTION FROM YEAST	Confirmation No.: 4661 Group Art Unit: 1636 Examiner: Michele K. Joike Attorney Docket: 2027.594096/RFE (2005941) **CUSTOMER NO. 23720**

REMARKS

Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

Sir:

This paper is submitted in response to the final Office Action dated October 18, 2007, for which the three-month date for response was January 18, 2008.

A request for a one-month extension of time to respond is included herewith along with the required fee. This one-month extension will bring the due date to February 18, 2008, which is within the six-month statutory period. Should such request or fee be deficient or absent, consider this paragraph such a request and authorization to withdraw the appropriate fee under 37 C.F.R. §§ 1.16 to 1.21 from Williams, Morgan & Amerson, P.C. Deposit Account No. 50-0786/2027.594096RE.

Remarks

The Examiner rejected claim 10 under 35 U.S.C. § 112, first paragraph, as allegedly lacking enablement for failure to comply with the biological material deposit requirements of 37 CFR 1.801-1.809. Applicants traverse this rejection.

Claim 10 recites (incorporating limitations from parent claims):

[A method of generating ascorbic acid, comprising a) obtaining a recombinant yeast capable of converting an ascorbic acid precursor into ascorbic acid, b) culturing the recombinant yeast in a medium comprising an ascorbic acid precursor, thereby forming ascorbic acid, and c) isolating the ascorbic acid], wherein the yeast is selected from *S. cerevisiae* strain GRF18U; *S. cerevisiae* strain W3031B; *K. lactis* strain PM6-7A; or *Z. bailii* strain ATCC 60483.

Therefore, the question is whether *S. cerevisiae* strain GRF18U; *S. cerevisiae* strain W3031B; *K. lactis* strain PM6-7A; and *Z. bailii* strain ATCC 60483 have been acceptably deposited or their deposit is not necessary. 37 CFR 1.802(b) makes clear that "[b]iological material need not be deposited, *inter alia*, if it is known and readily available to the public or can be made or isolated without undue experimentation."

S. cerevisiae strain GRF18U was deposited by coinventor Danilo Porro on July 31, 2000 with Agricultural Research Service Culture Collection (NRRL), Microbial Genomics and Bioprocessing Research Unit, National Center for Agricultural Utilization Research, 1815 North University Street, Peoria, Illinois 61604, USA. This strain was identified as deposit number NRRL Y-30320. NRRL is an International Depositary Authority under the Budapest Treaty. On August 2, 2001, Dr. Porro declared all restrictions on access to strain NRRL Y-30320 would be removed on grant of a patent on application 09/630,983, which is the parent of the present

application. Therefore, Applicant submits *S. cerevisiae* strain GRF18U has been deposited in compliance with 37 CFR 1.801-1.809.

S. cerevisiae strain W3031B is available from the American Type Culture Collection (ATCC) as deposit number ATCC 201238. The strain's availability from ATCC is demonstrated by the webpage http://www.atcc.org/common/catalog/numSearch/numResults.cfm, search conducted for ATCC number 201238, accessed by the undersigned on February 11, 2008, a copy of which is attached hereto. The webpage shows this strain is "known and readily available to the public." Therefore, no deposit is required (37 CFR 1.802(b)).

K. lactis strain PM6-7A is also "known and readily available to the public" as demonstrated by the many published references that cite Chen, *et al.*, Mol. Gen. Genet. **33:** 97–105 (1992) as the source of this strain. Chen, *et al.*, are coworkers of Wésolowski-Louvel, the first named author of Yeast 8, 711-719 (1992), the reference cited by the specification at p. 28, lines 3-5, as the source of this strain. References that cite Chen, *et al.*, include Tokunaga, *et al.*, *Yeast* **13**:699-706 (1997); Hansbro, *et al.*, *Curr. Genet.* **33**:46-51 (1998); Lemaire, *et al.*, *Genetics* **168**: 723-731 (2004); Šarinová, *et al.*, *Folia Microbiol.* **52**(3), 223-229 (2007); and Goffrini, *FEMS Yeast Res.* 7:180-187 (2007), copies of which are attached hereto. These references show that this strain was known and readily available to the public. Therefore, no deposit required. 37 CFR 1.802(b).

Z. bailii strain ATCC 60483 is available from the American Type Culture Collection (ATCC) as deposit number ATCC 60483. The strain's availability from ATCC is demonstrated by the webpage http://www.atcc.org/common/catalog/numSearch/numResults.cfm, search conducted for ATCC number 60483, accessed by the undersigned on February 11, 2008, a copy

of which is attached hereto. The webpage shows this strain is "known and readily available to the public." Therefore, no deposit is required. 37 CFR 1.802(b).

In conclusion, all the yeast strains recited by claim 10 are either acceptably deposited under 37 CFR 1.801-1.809 or their deposit is not necessary under 37 CFR 1.802(b). Applicants therefore request this rejection of claim 10 be withdrawn. Applicants submit all pending claims are in condition for allowance.

Respectfully submitted,

WILLIAMS, MORGAN & AMERSON, P.C.
CUSTOMER NO. 23720

February 15, 2008

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ATCC® Number: 60483™ Order this item **Price:** $185.00

Organism: *Zygosaccharomyces bailii* (Lindner) Guilliermond, teleomorph; deposited as *Saccharomyces bailii* Lindner, teleomorph

Designations: SG 242 [MB Y-24] **Isolation:** Imported citrus concentrate, the Netherlands

Depositors: HM Put

Biosafety Level: 1 **Shipped:** freeze-dried

Growth Conditions: ATCC medium 323: Malt agar medium
Temperature: 24.0C

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Comments:	heat-resistant [754]
Subcollection:	Yeasts
References:	754: Put HM , et al.. Heat resistance studies of yeasts; vegetative cells versus ascospores: erythromycin inhibition of sporulation in Kluyveromyces and Saccharomyces species. J. Appl. Bacteriol. 53: 73-79, 1982.

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Organism: *Saccharomyces cerevisiae* Meyen ex E.C. Hansen

Designations: W303-1B

Depositors: PP Slonimski **History:** ATCC<<--PP Slonimski <<--R. Rothstein

Biosafety Level: 1 **Shipped:** frozen

Genotype/ORF/ Gene Name: MATalpha leu2-3 leu2-112 trp1-1 ura3-1 his3-11 his3-15 ade2-1 can1-100 [25806]

Growth Conditions: ATCC medium 1245: YEPD
Temperature: 25.0C

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Related Products

Applications: transformation host [25806]

Mating Type: alpha

Karyotype: Ploidy: haploid

Subcollection: Yeasts

References: 25806: Remacha M , et al. Ribosomal acidic phosphoproteins P1 and P2 are not required for cell viability but regulate the pattern of protein expression in Saccharomyces cerevisiae. Mol. Cell. Biol. 15: 4754-4762, 1995. PubMed: 7651393

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A respiratory-deficient mutation associated with high salt sensitivity in *Kluyveromyces lactis*

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Keywords
salt resistance; respiratory deficiency; *Kluyveromyces lactis*; yeast; *BCS1*; *VMA13*.

Abstract

A salt-sensitive mutant of *Kluyveromyces lactis* was isolated that was unable to grow in high-salt media. This mutant was also respiratory-deficient and temperature-sensitive for growth. The mutation mapped in a single nuclear gene that is the ortholog of *BCS1* of *Saccharomyces cerevisiae*. The *BCS1* product is a mitochondrial protein required for the assembly of respiratory complex III. The *bcs1* mutation of *S. cerevisiae* leads to a loss of respiration, but, unlike in *K. lactis*, it is not accompanied by salt sensitivity. All the respiratory-deficient *K. lactis* mutants tested were found to be salt-sensitive compared to their isogenic wild-type strains. In the presence of the respiratory inhibitor antimycin A, the wild-type strain also became salt-sensitive. By contrast, none of the *S. cerevisiae* respiratory-deficient mutants tested showed increased salt sensitivity. The salt sensitivity of the *Klbcs1* mutant, but not its respiratory deficiency, was suppressed by the multicopy *KlVMA13* gene, a homolog of the *S. cerevisiae VMA13* gene encoding a subunit of the vacuolar H^+-ATPase. These results suggest that cellular salt homeostasis in *K. lactis* is strongly dependent on mitochondrial respiratory activity, and/or that the ion homeostasis of mitochondria themselves could be a primary target of salt stress.

Introduction

Yeast species are resistant, to various degrees, to high-salt environments. In the mechanisms of this resistance, we may distinguish two processes: (1) rapid primary response of the cell to dehydration; and (2) slow adaptation to the high-salt environments.

The first process, extensively studied in *Saccharomyces cerevisiae*, appears to be associated with increased turnover/accumulation of glycerol (or polyalcohol) and trehalose (Hohmann, 2002), and with the exclusion from the cell of a harmful solute such as NaCl, mediated by the P-type ATPase Ena1p and the sodium–proton antiporter Nha1p (Serrano *et al.*, 1997; Patterson *et al.*, 1999). The second process, which involves slow adaptation to high salt, is still poorly explored. In some cases, genomic rearrangements, such as gene amplification, may be involved (Prior *et al.*, 1996; Albrecht *et al.*, 2000). Studies of salt-sensitive mutations have revealed a wide range of associated phenotypes. In *S. cerevisiae*, the expression of at least 18 genes was strongly induced by high salt (Blomberg, 1995).

In the present work, to enable the study of salt sensitivity in *Kluyveromyces lactis* we selected mutants with high salt sensitivity. Among them, we found a new kind of mutant that showed, in addition to its sensitivity to high concentrations of NaCl, KCl and LiCl, a complete loss of respiratory activity. The mutation mapped in the *KlBCS1* gene, which codes for a mitochondrial protein involved in the assembly of respiratory complex III. A direct association of the salt sensitivity of yeast with a specific mitochondrial dysfunction has never been described previously. In the present work, the properties of the *Klbcs1* mutation and the association between salt sensitivity and mitochondrial dysfunction will be described.

Materials and methods

Strains, media and growth conditions

Table 1 lists the *K. lactis* and *S. cerevisiae* strains and derived mutants used in this study. *Escherichia coli* DH10B was used as a cloning host and for DNA propagation.

Complete medium (YP) contained 1% Bacto yeast extract (Difco) and 1% Bacto peptone (Difco). It was supplemented with a carbon source at 2% (glucose, glycerol or others as specified). Minimal medium contained 0.7% Yeast Nitrogen Base without amino acids (Difco), and 2% glucose, supplemented with appropriate auxotrophic requirements. For

Table 1. List of yeast strains

Strain	Genotype	Source
K. lactis		
PM6-7A	*MATa uraA1-1 adeT-600*	Chen *et al.* (1992)
PM6-7A/A16	*MATa uraA1-1 adeT-600 Klbcs1*	This study
PM6-7A/ΔKlcyc1 (KG6)	*MATa uraA1-1 adeT-600, Klcyc1::URA3*	Chen & Clark-Walker (1993)
MW179-1D	*MATα uraA1-1 leu2 lac4-8 trpA1 ade1*	M. Wésolowski-Louvel (University of Lyon 1)
MW179-1D/ΔKlcox14	*MATα uraA1-1 leu2 lac4-8 trpA1 ade1 Klcox14::kanMX4*	Fiori *et al.* (2000)
JBD100	*MATα trp1 lac4-1 ura3*	Heus *et al.* (1990)
JBD100/Klcox18 (M5)	*MATα trp1 lac4-1 ura3 Klcox18*	Hikkel *et al.* (1997)
JBD100/Klcytc1 (M3)	*MATα trp1 lac4-1 ura3 Klcytc1*	Gbelská *et al.* (1996)
WMH9802/ΔKlqcr8	*MATα uraA1-1 leu2 lac4-8 trpA1 ade1 Klqcr8::URA3*	Brons *et al.* (2001)
2360/7	*MATα lysA*	Parma collection
S. cerevisiae		
W303-1A	*MATa SUC2 ade2 can1 his3 leu2 trp1 ura3*	R. Rothstein (Columbia University)
W303-1A/Δbcs1	*MATa SUC2 ade2 can1 his3 leu2 trp1 ura3 bcs1::HIS3*	Nobrega *et al.* (1992)
BY4741	*MATa ura3Δ0 his3Δ1 leu2Δ0 met15Δ0*	Euroscarf collection
BY4741/Δsop1 (YPR032W)	*MATa ura3Δ0 his3Δ1 leu2Δ0 met15Δ0 sop1:: kanMX4*	Euroscarf collection
MH41-7B *rho*$^+$ and *rho*$^-$ derivatives*	*MATa ade2 his1*	Institut Curie, Orsay
MH32-12D *rho*$^+$ and *rho*^{0} derivatives*	*MATa ade2 his1*	Institut Curie, Orsay
IL8-8C/HF71/*rho*^{0}	*MATα trp1 his1, rho*^{0}	Institut Curie, Orsay
IL125-10C/*rho*^{0}	*MAT α ura, rho*^{0}	Institut Curie, Orsay

*MH41-7B/HF21 *rho*^{0}, MH41-7B/OI-3 *rho*$^-$, MH41-7B/C7 *rho*$^-$, MH41-7B/P1 *rho*$^-$, MH32-12D/*rho*^{0} (Wésolowski-Louvel & Fukuhara, 1979)

plate tests, these media were solidified with 2% agar. Salt resistance/sensitivity was tested on YP-glucose plates containing the indicated concentrations of salts or sugars. The culture temperature was 28 °C unless specified otherwise. Antimycin A (Sigma) was used at a concentration of 5 μM throughout. Genetic procedures for mating and sporulation were done on ME plates (5% malt extract, 3% Bacto agar).

Isolation of mutants

Yeast cells were mutagenized with UV irradiation according to Wésolowski-Louvel *et al.* (1992). Cells at a density of 10^8 cells mL^{-1} were exposed to 75 J m^{-2} of UV radiation. Survival was 20–30%. Cells were plated for single colonies on YP-glucose, and replica-plated on NaCl-containing medium. Putative mutants (negative growth on 1.5 M NaCl) were subcloned and retested for their salt-sensitive phenotype.

Cytochrome absorption spectra

Cells, grown to early stationary phase on YP medium supplemented with 2% glucose, were harvested by centrifugation, washed twice with cold (4 °C) distilled water, and suspended in a volume of cold water twice the pellet volume. Differential spectra between reduced and oxidized cells were recorded at room temperature using a Cary 219 spectrometer. The bandwidth was 1 nm and the scan speed was 0.5 nm s^{-1}. The cell suspension was reduced by sodium dithionite.

General methods

Published procedures were used for the transformation of *K. lactis* (Bianchi *et al.*, 1987) and *E. coli* (Mandel & Higa, 1970). DNA manipulation, restriction enzyme digestion, plasmid engineering and standard techniques were performed according to Sambrook & Russel (2001). Sequencing was performed using a Beckman CEQ2000 automatic sequencer. Sequence analysis was performed with the BLASTP program (Altshul *et al.*, 1990), and sequence alignment with the CLUSTAL W program (Thompson *et al.*, 1994). The GenBank accession numbers for *KlBCS1* and for *KlVMA13* are **AJ299738** and **AJ547613**, respectively.

The amplification of the mutated *Klbcs1* allele was obtained by PCR with PM6-7A/A16 genomic DNA as a template and the primer pair KlBCS1F, 5′-AATCCGAGGCCTCGATTTCC-3′, and KlBCS1R, 5′-GGATGGACAACGAACGATAT-3′.

Results

Isolation and phenotypic characterization of mutants of *K. lactis* with high salt sensitivity

The strain PM6-7A was UV-mutagenized, and about 25 000 cells were plated on YP-glucose plates. The colonies were replica-plated onto YP-glucose containing 1.5 M NaCl. Many colonies showed slow, leaky growth. Only three colonies (A8, A10 and A16) were clearly incapable of growing on the high-salt plates, as shown in Fig. 1. They

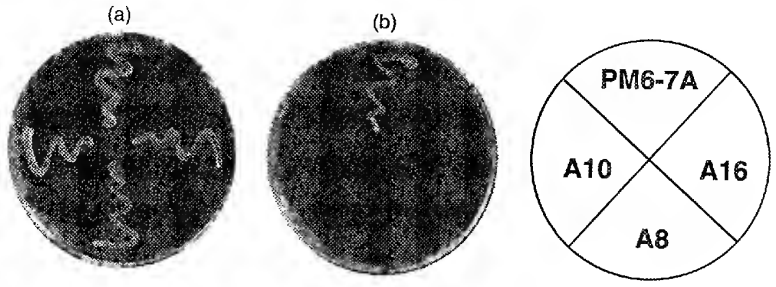

Fig. 1. Salt sensitivity of *Kluyveromyces lactis* mutants. Strain PM6-7A and the three salt-sensitive mutants derived from it were streaked on YP-glucose plates with (b) or without (a) 1.5 M NaCl, and allowed to grow at 28 °C for 2 days.

Table 2. Salt sensitivity of three mutants

Strain	NaCl 1.5 M	KCl 1.5 M	LiCl 0.3 M	Glucose 2 M	Sorbitol 2 M	Sucrose 1 M
PM6-7A	+	+	+	+	+	+
PM6-7A/A8	–	+	+/–	+	+	+
PM6-7A/A10	–	+	–	+	+	+
PM6-7A/A16	–	–	–	+	+	+

The three mutants, A8, A10 and A16, are subclones of PM6-7A obtained by UV mutagenesis. All the tests were performed on YP-glucose plates supplemented with high-osmolarity solutes as indicated. The signs + and – indicate the occurrence and absence of growth, respectively, as recorded after 3 days of incubation.

were submitted to further tests of osmosensitivity on high-salt and high-sugar media. The results are shown in Table 2.

All the mutants were sensitive to 1.5 M NaCl, but not to high concentrations of sugars. They could be distinguished by their different sensitivities to 1.5 M KCl and to 300 mM LiCl. In particular, a mutant, called A16, was sensitive to all three salts, and moreover its growth was sensitive to high temperature (36 °C) on YP-glucose. This mutant was also unable to grow on nonfermentable substrates (glycerol, ethanol and lactate). It was indeed respiratory-deficient, displaying an 80-fold decrease in oxygen consumption rate compared to the wild-type parental strain (data not shown). The cytochrome absorption spectra (Fig. 2) indicated that cytochrome *b* and cytochrome $a+a_3$ were reduced by 25% and 65%, respectively. Our study focused on the particular pleiotropic mutant A16.

The A16 mutant appears to have a single gene mutation, as suggested by two observations: (1) all spontaneous back mutations restored a complete wild-type phenotype, and (2) transformation of the mutant with a single gene (*KlBCS1*), as described below, fully complemented all the deficient phenotypes of A16. Although genetic crosses with wild-type laboratory strains gave diploids severely impaired in sporulation, we were able to obtain a diploid able to sporulate by crossing the mutant with the 2360/7 strain. Tetrad analysis demonstrated that the pleiotropic phenotype was due to a single nuclear mutation, as we obtained a 2 : 2 Mendelian segregation.

Cloning and characterization of the *KlBCS1* gene

The A16 mutant was transformed with a *K. lactis* genomic library constructed on a centromere-based vector KCp491

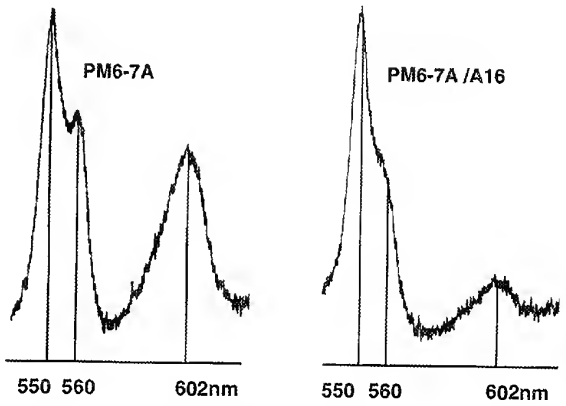

Fig. 2. Cytochrome spectra of *Kluyveromyces lactis* PM6-7A and its respiratory-deficient mutant A16. The absorbance peaks at 550, 560 and 602 nm correspond to cytochrome c, cytochrome *b* and cytochromes $a+a_3$, respectively.

carrying a *URA3* marker (Prior *et al.*, 1993). The Ura^+ transformants were tested for their salt sensitivity. Among them, two clones were capable of growing on 1.5 M NaCl. The Ura^+ phenotype and the salt resistance cosegregated in the course of spontaneous loss of the marker. The two transformants also recovered temperature resistance and respiratory competence. Both carried an identical plasmid (named pOSME) that contained a DNA insert of 6.0 kb. Fragments of this DNA were subcloned, and a segment spanning a 2.7-kb *Eco*RI fragment (carried by a plasmid named pOSME/E27) was found to be responsible for the transformed phenotype. The insert contained a putative single ORF of 1.35 kb. The predicted product of this DNA

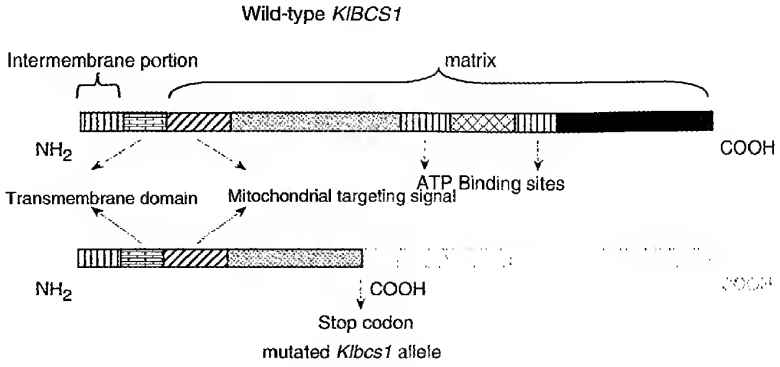

Fig. 3. Structure of KlBcs1p as deduced from the DNA sequence. The amino acid sequence of the *KlBCS1* product was deduced from the nucleotide sequence. Comparison with the *Saccharomyces cerevisiae* ortholog (Nobrega *et al.*, 1992) clearly identified characteristic sequence motifs as indicated. The lower half of the figure shows the DNA sequence-deduced structure of KlBcs1p in the A16 mutant.

was a 450-amino-acid protein that showed 69% identity with the Bcs1p of *S. cerevisiae*. This is a nuclear-encoded mitochondrial protein of the AAA family (ATPase associated with diverse cellular activities) that in *S. cerevisiae* controls the assembly of the cytochrome bc_1 complex and leads to a total absence of complex III activity. It has been proposed that Bcs1p acts as an ATP-dependent chaperone maintaining the precomplex in a competent state for the subsequent assembly of Rieske FeS and Qcr10p proteins (Nobrega *et al.*, 1992; Cruciat *et al.*, 1999).

The complex III deficiency in the *bcs1* mutant also affects the amount of complex IV assembly and activity. In the cytochrome absorption spectra of the *Klbcs1* mutant (Fig. 2), the effect on cytochromes $a+a_3$ of complex IV was more evident; the effect on cytochrome *b* was less pronounced but distinguishable from that of the wild type. With regard to cytochrome c_1, the negative effect of the mutation is evident from the reduced shoulder on the right side of the peak at 550 nm. Taken together, these results indicated that there was the same pleiotropic effect on cytochrome assembly in the *K. lactis bcs1* mutant as was observed in the *S. cerevisiae* mutant.

As expected, the structural organization of the cloned *K. lactis* gene was similar to that of *BCS1*, showing the presence of two supposed ATP-binding motifs and a mitochondria-targeting signal (Fig. 3).

The mutant allele of the A16 strain was also sequenced by means of triplicate PCR amplification of the mutant DNA. The mutation corresponded to the introduction of a stop codon in the middle of the gene, resulting in a large deletion of the C-terminal half of the protein, with a loss of the putative ATP-binding motifs (Fig. 3).

Functional complementation of *S. cerevisiae bcs1* mutation by the cloned *K. lactis* gene

Given the structural similarity of the cloned gene to *BCS1*, we carried out a functional complementation experiment. The cloned *K. lactis* DNA on the KCp491 vector that can also replicate in *S. cerevisiae* was transformed into the *S. cerevisiae* Δ*bcs1* mutant. The transformants fully recovered the ability to grow on nonfermentable carbon sources (Table 3). The *K. lactis* gene was therefore named *KlBCS1*, and the mutant allele *Klbcs1*. Having observed a functional homology between *KlBCS1* and *BCS1*, we wanted to know whether in *S. cerevisiae* the *BCS1* gene is involved in salt resistance. In contrast to what was observed in *K. lactis*, the *S. cerevisiae* Δ*bcs1* mutant did not show increased sensitivity to 1 M NaCl compared to its isogenic wild type (1 M was the highest concentration tolerated by the wild-type *S. cerevisiae* strains used here, as compared to 1.5 M for *K. lactis* strains) (Table 3). Moreover, the mutant was also not temperature-sensitive for growth. Thus the phenotypes of the *bcs1* mutation clearly differed between the two species.

Does the salt-sensitive phenotype always accompany the respiratory deficiency in *K. lactis*?

In order to know whether the salt sensitivity was due to the *Klbcs1* mutation *per se* or to the respiratory deficiency resulting from the mutation, we examined the salt sensitivity of available *K. lactis* respiratory-deficient mutants. Several respiratory-deficient mutants of *S. cerevisiae* were also included for comparison. The results obtained (Table 3) indicated that all the respiratory-deficient mutants of *K. lactis* were sensitive to 1.5 M NaCl (Fig. 4). These were mutants of cytochrome *c*, cytochrome oxidase subunits and complex III subunits, respectively. One exception was a cytochrome c_1 mutant that grew on the high-salt medium (see Discussion). The strong correlation between respiratory deficiency and salt sensitivity was further supported by the observation that the wild-type *K. lactis* strains became salt-sensitive in the presence of the respiratory inhibitor antimycin A (Table 3). By contrast, none of the respiratory-deficient mutants of *S. cerevisiae*, including *bcs1*, were salt-sensitive compared to isogenic wild-type strains. As a

Table 3. Respiratory deficiency and salt sensitivity: comparison between *Kluyveromyces lactis* and *Saccharomyces cerevisiae*

	YP-glycerol	YP-glucose+NaCl
K. lactis		
PM6-7A	+	+
PM6-7A/A16	–	–
PM6-7A/A16+[*KlBCS1*]	+	+
PM6-7A/ΔKlcyc1	–	–
MW179-1D	+	+
MW179-1D/ΔKlcox14	–	–
WMH9802/ΔKlqcr8	–	–
JBD100	+	+
JBD100/Klcox18	–	–
JBD100/Klcytc1	–	+
PM6-7A+antimycin A	–	–
MW179-1D+antimycin A	–	–
JBD100+antimycin A	–	–
S. cerevisiae		
W303-1A	+	+
W303-1A/Δbcs1	–	+
W303-1A/Δbcs1+[*KlBCS1*]	+	+
MH41-7B, *rho*$^+$	+	+
MH41-7B/HF21, *rho*^{0}	–	+
MH41-7B/OI-3, *rho*$^-$	–	+
MH41-7B/C7, *rho*$^-$	–	+
MH41-7B/P1, *rho*$^-$	–	+
MH32-12D, *rho*$^+$	+	+
MH32-12D/*rho*^{0}	–	+
IL8-8C/HF71, *rho*^{0}	–	+
IL125-10C/*rho*^{0}	–	+
BY4741	+	+
BY4741/Δsop1	+	–
W303-1A+antimycin A	–	+
BY4741+antimycin A	–	+

Kluyveromyces lactis and *Saccharomyces cerevisiae* strains were streaked on YP plates containing glycerol (test for respiratory competence) or glucose and NaCl (test for salt sensitivity), as indicated. +[*KlBCS1*] indicates the presence of the monocopy plasmid carrying the *KlBCS1* gene. Note that for *S. cerevisiae*, the salt sensitivity test was performed on 1 M NaCl, the maximal salt concentration tolerated by the wild-type strains, as compared to 1.5 M for *K. lactis*. The *K. lactis* wild-type strains used here cease to grow on 1.7 M NaCl.

negative control, we used the *sop1* mutant of *S. cerevisiae*, a well-known salt-sensitive strain (Larsson *et al.*, 1998).

A multicopy suppressor of *Klbcs1*

The role of KlBcs1p in salt resistance is not obvious. In order to find possible linked elements, we looked for a multicopy suppressor of *Klbcs1* mutation. The *Klbcs1* mutant was transformed with a *K. lactis* genomic library carried by the multicopy *K. lactis*/*S. cerevisiae* shuttle vector pSK1 (Wésolowski-Louvel *et al.*, 1988). Among the 6000 Ura$^+$ transformants, one single clone recovered the ability to grow on 1.5 M NaCl (in YP-glucose). However, this clone remained respiratory-deficient and temperature-sensitive for growth. Therefore, the suppressor appeared to be extragenic. The suppressed clone contained a plasmid with a DNA insert of about 6 kb. The predicted product of the gene found in this segment has an identity of 43% with the protein encoded by the *VMA13* gene of *S. cerevisiae*. The Vma13p of *S. cerevisiae* has been known to form part of the vacuolar H^+-ATPase complex (V-ATPase), an ATP-dependent proton pump that acidifies the vacuolar compartment. Vma13p is thought to be an activator or a stabilizer of the multimeric V-ATPase complex (Anraku *et al.*, 1992). The functional equivalence of *KlVMA13* and *VMA13* was confirmed by complementation of the *vma13* mutation by *KlVMA13* (recovery from Ca^{2+} sensitivity of the mutant; data not shown).

A contribution of V-ATPase to the mechanism of salt tolerance in yeast has been reported (Hamilton *et al.*, 2002). ATP hydrolysis is coupled with active proton transport inside the vacuole, thus generating a chemical gradient that drives transport of ions such as Na^+ and Ca^{2+}, which is mediated by the Na^+/H^+ antiporter, NHX1 (Nass & Rao, 1998; Hirata *et al.*, 2002).

If the observed suppression resulted from a major role played by V-ATPase in the ionic homeostasis of mitochondria, we might expect that the salt-sensitive phenotype of other *K. lactis* respiratory-deficient mutants might also be complemented by the multicopy *KlVMA13* gene. However, this was not the case. The suppressor effect of *KlVMA13* was specific for the *Klbcs1* mutation, suggesting that if the V-ATPase contributes to the ionic balance of the mitochondria, this might occur through a mechanism involving Bcs1p.

Discussion

Relationship between respiratory deficiency and salt resistance in *K. lactis*

Salt resistance involves many genes. Studies on several yeast species, in particular *S. cerevisiae*, have led to the identification of different types of mutation that display different phenotypes. A striking finding was the identification of *BCS1* as a genetic determinant involved in salt resistance in *K. lactis*. Because it is difficult to imagine a specific direct involvement of Bcs1p in salt resistance, we hypothesized that the salt sensitivity of the *Klbcs1* mutant may not be a specific phenotype of this particular mutation, but rather a general consequence of the respiratory deficiency. The observation that wild-type *K. lactis* strains became salt-sensitive when respiration was specifically blocked by antimycin A was in favor of this interpretation. When we investigated the osmotic response of other respiratory-deficient mutants of *K. lactis*, all of them, as expected, were salt-sensitive, except

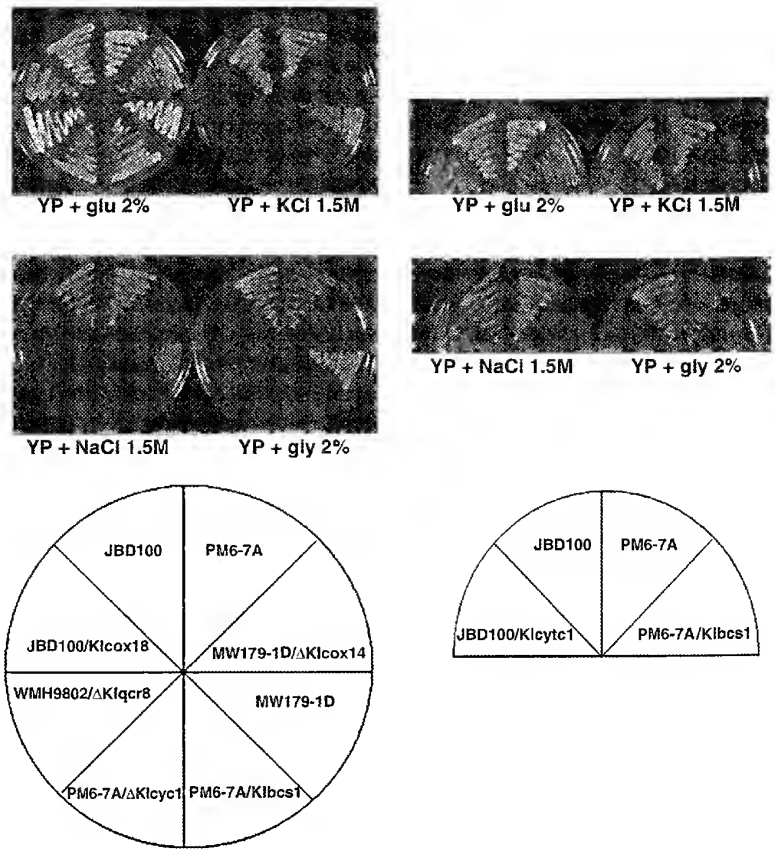

Fig. 4. Salt-sensitive phenotype of *Kluyveromyces lactis* respiratory-deficient mutants. The salt sensitivity of various respiratory mutants of *K. lactis* was tested against either 1.5 M NaCl or 1.5 M KCl included on YP-glucose plates. The respiratory deficiency is shown by their absence of growth on YP-glycerol plates.

for a cytochrome c_1 mutant that maintained the resistant phenotype of the parental strain. However, this mutant retained 20% of the respiratory capacity of its wild-type parent (Gbelská *et al.*, 1996). Thus, this mutant may not be considered to be strictly respiration-negative. For this reason, it should not be considered an exception to the relationship between respiratory deficiency and salt sensitivity. Therefore, the results with all the respiratory-deficient mutants, as well as those obtained with antimycin A, indicate that the lack of respiratory activity was correlated with the salt sensitivity.

Difference between *K. lactis* and *S. cerevisiae*

Unlike in *K. lactis*, in *S. cerevisiae* the *bcs1* mutant as well as all other respiratory-deficient mutants retain high salt resistance, which is similar to the level observed in the wild-type strains. Therefore, the two yeast species clearly differ by the presence/absence of a link between respiratory deficiency and salt sensitivity. A possible reason could be that the laboratory strains of *S. cerevisiae* originate mainly from fermentation media, and hence these strains show a preference for a fermentative life rather than a respiratory mode of growth. We would expect such yeasts to possess stress-resisting mechanisms that do not require respiratory metabolism. Conversely, *K. lactis* strains have a strong respiratory activity, and normally this microorganism has a respiratory mode of life. The reducing potential generated by its strong glucose 6-phosphate shunt has to be recycled by active respiratory activity. Therefore, this species may have developed a stress response mechanism that is more tightly associated with mitochondrial functions, in comparison with *S. cerevisiae*. A respiratory deficiency or a mitochondrial mutation may then result in increased sensitivity to certain stresses. Such an interpretation, perhaps oversimplified, can be experimentally tested by the use of other yeast species showing a high dependence of growth on respiratory activity. In those species, salt resistance may also be linked to active mitochondrial functions, and in this regard, the responses of *S. cerevisiae* to high salt may reflect an exceptional physiology of this species.

Finally, the specific suppression of multicopy *KlVMA13* in the *Klbcs1* mutation has not yet been explained. Moreover, a relationship of V-ATPase with the mitochondrial system has been reported by Ohya *et al.* (1991), who observed that some of the mutations of the V-ATPase complex were accompanied

by a respiratory-deficient phenotype in *S. cerevisiae*. Recently, links between iron and copper metabolism and mitochondrial and vacuolar function have also been found. In particular, a role for *VMA13* in metal trafficking has been demonstrated (van Bakel *et al.*, 2005).

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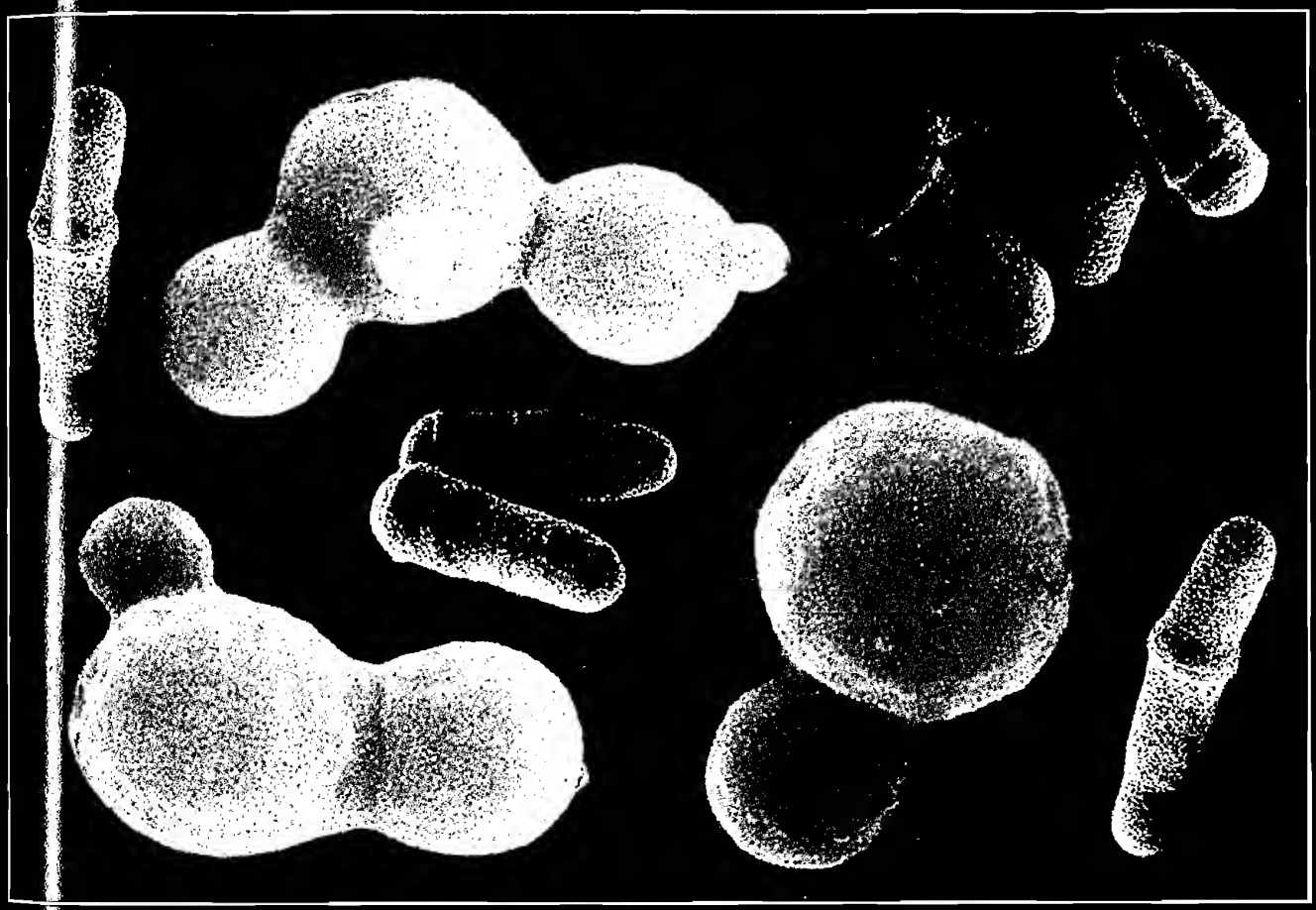

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Secretion of Mouse α-Amylase from *Kluyveromyces lactis*

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We constructed two mouse α-amylase secretion vectors for *Kluyveromyces lactis* using the well-characterized signal sequence of the pGKL 128 kDa killer precursor protein. Both *PHO5* and *PGK* expression cassettes from *Saccharomyces cerevisiae* directed the expression of mouse α-amylase in YPD medium at a similar level of efficiency. *K. lactis* transformants secreted glycosylated and non-glycosylated α-amylase into the culture medium and both species were enzymatically active. The *K. lactis*/*S. cerevisiae* shuttle secretion vector pMI6 was constructed, and *K. lactis* MD2/1(pMI6) secreted about four-fold more α-amylase than *S. cerevisiae* YNN27 harboring the same plasmid, indicating that *K. lactis* is an efficient host cell for the secretion and production of recombinant proteins.

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KEY WORDS — Glycosylation; leader-peptide; signal sequence

INTRODUCTION

Yeast is a very useful host in which to express foreign genes, since it is a safe eukaryotic microorganism with well-established fermentation technology for large-scale production (Romanos *et al.*, 1992). Since the application of recombinant DNA techniques ranges from medical therapeutics to recombinant enzyme production for food science and for biomass utilization, the low-cost production of useful recombinant proteins has become important in recent biotechnology. A large amount of yeast cells can be easily grown by high density cultivation at lower cost than any other eukaryotic expression system.

We have been investigating highly efficient secretion systems for foreign gene products in *Saccharomyces cerevisiae* (Tokunaga *et al.*, 1987, 1988; Kanaya *et al.*, 1989) and in the fission yeast *Schizosaccharomyces pombe* (Tokunaga *et al.*, 1993). Recently, non-*Saccharomyces* (or alternative) yeasts such as *Pichia pastoris*, *Hansenula polymorpha* and *Yarrowia lipolytica* have been applied as new host cells (Romanos *et al.*, 1992). Among alternative yeast systems, *Kluyveromyces lactis* has much potential as a host, since it has been used to produce β-galactosidase for the food industry. Furthermore, many types of expression vehicles, such as 2 μm-like multi-copy pKD1 derivatives, integration and centromeric vectors have been developed (Wésolowski-Louvel *et al.*, 1996). The secretion of high levels of bovine prochymosin (van den Berg *et al.*, 1990), human serum albumin (Fleer *et al.*, 1991a) and human interleukin-1β (Fleer *et al.*, 1991b) from *K. lactis* cells has been described.

In this study, we describe the efficient expression and secretion of mouse α-amylase from *K. lactis* MD2/1 cells into culture medium. *K. lactis* cells secreted both glycosylated and non-glycosylated α-amylases into the culture medium and both were proven to be enzymatically active by staining after non-denaturing gel electrophoresis. Furthermore,

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Table 1. Description of strains and plasmids used in this study.

Strains and plasmids	Characteristics
Strains	
K. lactis	
MD2/1	*MATα, uraA, argA1, lysA1, rag1, rag2, cir*$^+$
PM6-7A	*MATa, uraA, ade2, cir*$^+$
S. cerevisiae	
YNN27	*MATα, trp1, ura3, cir*$^+$
S150-2B	*MATa, his3, leu2, trp1, ura3, cir*$^+$
W303-1B	*MATα, his3, leu2, trp1, ura3, ade2, cir*$^+$
Plasmids	
pSPHO4	Expression vector for *K. lactis* using *PHO5* promoter-terminator cassette of *S. cerevisiae* and secretion signal from 128 kDa killer precursor protein
pSPGK1	Expression vector for *K. lactis* using *PGK* promoter-terminator cassette of *S. cerevisiae* and secretion signal from 128 kDa killer precursor protein
pSK1	*K. lactis*/*S. cerevisiae* shuttle vector
pSMF38TMA	Mouse α-amylase secretion vector for *S. cerevisiae*
pKA128	Mouse α-amylase secretion vector constructed from pSPHO4
pMI5	Mouse α-amylase secretion vector constructed from pSPGK1
pMI6	Mouse α-amylase secretion vector constructed from pSK1

we directly compared the secretion efficiency of mouse α-amylase from *K. lactis* and *S. cerevisiae* cells using the *K. lactis*/*S. cerevisiae* shuttle vector. We found that *K. lactis* MD2/1 cells harboring the expression shuttle vector pMI6 secreted about four-fold more mouse α-amylase into the culture medium than *S. cerevisiae* YNN27 transformants.

MATERIALS AND METHODS

Yeast strains and medium

The strains and plasmids used in this study are summarized in Table 1. The media were YPD (1% Bacto yeast extract, 2% Bacto peptone and 2% glucose), YP (1% Bacto yeast extract and 2% Bacto peptone) and SC (0·67% yeast nitrogen base, amino acid mixture and 2% glucose; Sherman *et al.*, 1986). YPS (1% Bacto yeast extract, 2% Bacto peptone, 1% starch and 2% agar) plates were used for halo assays of α-amylase activity (Tokunaga *et al.*, 1987). For the tunicamycin treatment of the cells, *K. lactis* transformants were cultured at 30°C in YPD medium containing 0·5 μg/ml of tunicamycin. Plasmids were constructed in *Escherichia coli* HB101. *E. coli* transformants were selected on LB plates containing 100 μg/ml ampicillin.

Construction and transformation of secretion vectors

DNA manipulation was as described by Maniatis *et al.* (1982).

Plasmids were constructed as shown in Figure 1A. The expression and secretion vectors, pSPHO4 and pSPGK1, have been described previously (Wéslowski-Louvel *et al.*, 1996). The plasmid pSMF38TMA (Nishizawa *et al.*, 1987) was digested with *Bam*HI and the 1·4 kb fragment containing the α-amylase gene without a signal sequence was digested with mung-bean nuclease. This fragment was subcloned into the pSPHO4 *Eco*RI site that was digested with mung-bean nuclease and bacterial alkaline phosphatase.

The construction of plasmid pMI5 is also shown in Figure 1A. The same 1·4 kb fragment containing the α-amylase gene was subcloned into the *Eco*RI site of pSPGK1, that was digested with mung-bean nuclease and bacterial alkaline phosphatase.

Plasmid pMI5 was digested with *Bam*HI and *Sal*I to isolate a fragment containing the *PGK* promoter, the signal sequence of the 128 kDa killer precursor protein, the mouse α-amylase gene without its own signal sequence and the *PGK*

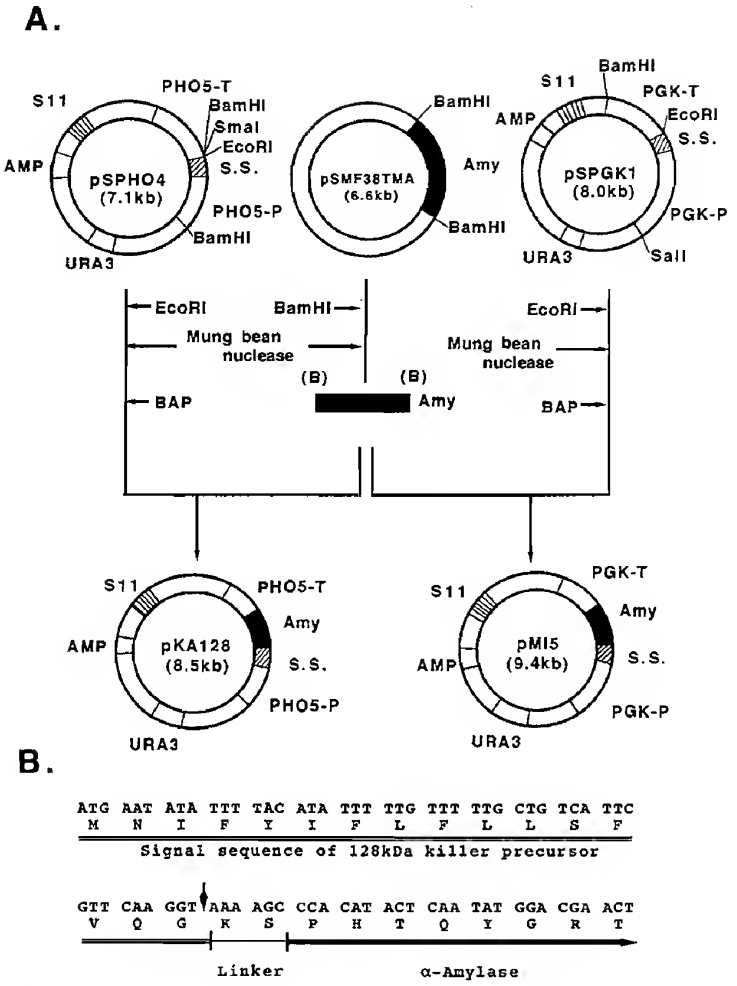

Figure 1. Construction of secretion plasmids pKA128 and pMI5. (A) Details are described in Materials and Methods. Expression vectors, pSPHO4 and pSPGK1, have been described previously (Wésolowski-Louvel *et al.*, 1996). PHO5-P, promoter of *PHO5*; S.S., signal sequence of the 128 kDa killer precursor protein; PHO5-T, terminator of *PHO5*; S11, replication origin of pKD1; AMP, ampicillin resistance; Amy, mouse α-amylase; PGK-P, promoter of *PGK*; PGK-T, terminator of *PGK*; BAP, bacterial alkaline phosphatase. (B) N-terminal sequence of α-amylase in secretion vectors, pKA128, pMI5 and pMI6. Double underline shows signal sequence derived from the gene encoding 128 kDa killer precursor protein. Underline shows the linker sequence and the thick arrow represents the α-amylase gene. The 19th Pro residue in the present secretion vector corresponds to the 19th residue of mouse salivary α-amylase precursor protein.

transcription termination signal. This fragment was subcloned into *Bam*HI-*Sal*I-digested pSK1 (the *K. lactis*/*S. cerevisiae* shuttle vector; Prior *et al.*, 1993) to construct pMI6.

The yeast was transformed using the Li^+-salt described by Ito *et al.* (1983).

Assay of α-amylase activity

For the halo assay, yeast transformants were patched onto YPS plates and incubated for 48–72 h at 30°C. Halos surrounding the transformants were detected by the absence of iodo-staining (I_2-KI) of the digested starch (Tokunaga *et al.*, 1993).

The activity of mouse α-amylase secreted into the medium was determined as described by Nelson (1944) and Somogyi (1952). Transformants were precultured in SC medium and 0·5 ml of this seed culture was inoculated onto 5 ml of rich medium (YP plus carbon source) for the production and secretion of α-amylase. The reaction mixture containing 0·5 ml of 1% soluble starch dissolved in 15 mM-N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) buffer (pH 7·0) with 1·5 mM-$CaCl_2$ and 0·5 ml of crude enzyme (culture supernatant dialysed against 15 mM-HEPES buffer, pH 7·0) were incubated at 37°C. At the appropriate time, 0·2 ml of reaction mixture was mixed with the Somogyi solution, then the Nelson solution, as described (Nelson, 1944; Somogyi, 1952).

Active staining of α-amylase on non-denatured polyacrylamide gel electrophoresis (PAGE) proceeded as follows. Crude enzyme was resolved on 6·5% polyacrylamide non-denatured gels with 25 mM-Tris/192 mM-glycine buffer (pH 8·3) at 20 mA for 6 h at 4°C. The gel after electrophoresis was incubated in 50 mM-HEPES buffer (pH 7·0) containing 0·02% soluble starch for 1 h at room temperature, then stained with 0·2% KI–2% I_2.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting

Proteins were separated by sodium dodecyl sulfate (SDS)–PAGE as described by Laemmli (1970). Proteins resolved on SDS–PAGE were electroblotted onto a nitrocellulose membrane using a semi-dry blotting apparatus (BIO CRAFT BE-300) and a buffer of 25 mM-Tris/192 mM-glycine/20% methanol, pH 8·3. The blotted α-amylase was immunostained with an anti-human-α-amylase IgG/peroxidase-conjugated anti-rabbit-IgG antibody/4-chloronaphthol-H_2O_2 (Tokunaga *et al.*, 1983, 1987, 1988).

RESULTS AND DISCUSSION

Expression and secretion of mouse α-amylase from K. lactis *transformants*

We constructed α-amylase secretion vectors using the signal peptide of pGKL killer 128 kDa precursor protein and multi-copy expression vectors (Figure 1). This signal sequence has been applied to *S. cerevisiae* (Tokunaga *et al.*, 1987), *Sz. pombe* (Tokunaga *et al.*, 1993) and *K. lactis* (Fleer *et al.*, 1991a,b) recombinant systems.

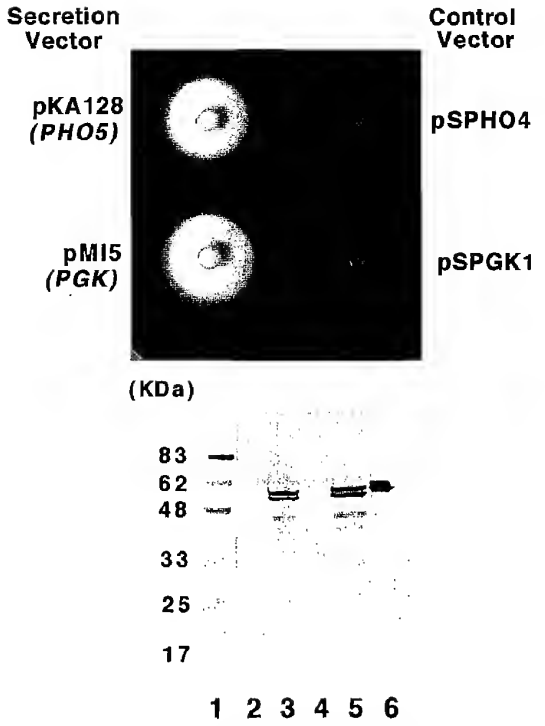

Figure 2. Halo assay and immunoblotting of secreted mouse α-amylase. (a) Halo assay of pKA128 and pMI5. Controls without the α-amylase gene are also shown. (b) Immunoblotting of secreted mouse α-amylase. Proteins in the culture supernatant (1·5 ml) were concentrated by 10% trichloroacetic acid precipitation, resolved by SDS–PAGE, then immunoblotted. Lane 1, pre-stained marker; lane 2, *K. lactis* (pSPHO4); lane 3, *K. lactis* (pKA128); lane 4, *K. lactis* (pSPGK1); lane 5, *K. lactis* (pMI5); lane 6, authentic human salivary α-amylase (Sigma IX-A), the antigen used for preparing anti-α-amylase antibody.

The secretion of mouse α-amylase from *K. lactis* MD2/1 cells is shown in Figure 2. *K. lactis* cells harboring plasmids pKA128 and pMI5 formed clear halos on YPS plates, indicating that *K. lactis* can secrete α-amylase into the culture medium using the signal peptide of the pGKL killer 128 kDa precursor protein (Figure 2a). The size of the *K. lactis* (pKA128) halo was similar to that of *K. lactis* (pMI5), suggesting that *PHO5* and *PGK* expression cassettes of *S. cerevisiae* function sufficiently well to support good levels of secretion when used with a suitable leader sequence in *K. lactis* transformants. *K. lactis* harboring the vector plasmid pSPHO4 and pSPGK1 without the α-amylase gene did not produce halos. We also constructed α-amylase secretion vectors using the signal peptide of pGKL killer 28 kDa precursor protein, but the efficiency of secretion was lower

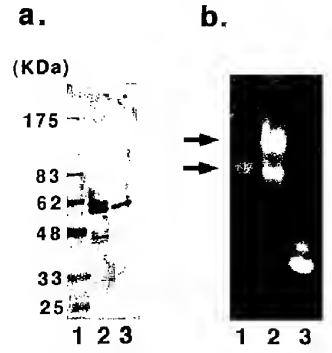

Figure 3. Immunoblotting and active staining of secreted mouse α-amylases with and without tunicamycin. (a) Immunoblotting of α-amylase. Lane 1, prestained marker; culture with (lane 3) and without (lane 2) 0·5 μg/ml tunicamycin. (b) Active staining of α-amylases. *K. lactis* (pMI5) cultured with (lane 1) and without (lane 2) tunicamycin. Lane 3, authentic human salivary α-amylase.

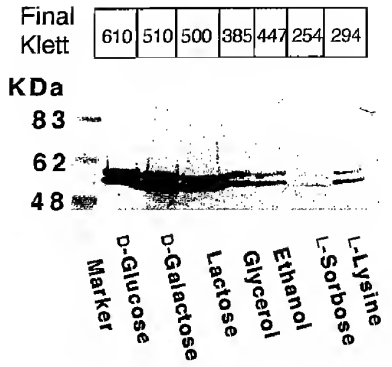

Figure 4. Effects of carbon sources on α-amylase secretion. Immunoblotting of secreted mouse α-amylase. Proteins in the culture supernatant (1·5 ml) were concentrated by 10% trichloroacetic acid precipitation, then resolved by SDS–PAGE and immunoblotted. The final cell growth (Klett value) is also shown.

than that of the 128 kDa protein (data not shown) as found in *Sz. pombe* transformants (Tokunaga *et al.*, 1993).

We identified α-amylase secreted into the culture medium by means of Western blotting using anti-human-α-amylase antibody. As shown in Figure 2b, culture supernatants of *K. lactis* (pKA128) and *K. lactis* (pMI5) contained two bands with a molecular mass of around 55 000, which cross-reacted with anti-human α-amylase antibody (lanes 3 and 5). No bands were evident in *K. lactis* cells containing pSPHO4 or pSPGK1 (lanes 2 and 4).

Glycosylation of secreted α-amylase

It has been reported that recombinant mouse α-amylase secreted into the culture medium by *S. cerevisiae* is composed of glycosylated and non-glycosylated molecules (Tokunaga *et al.*, 1988, 1992). To clarify the glycosylation status of α-amylase, *K. lactis* cells were grown in the presence of tunicamycin (0·5 μg/ml culture) at 30°C overnight. The α-amylase secreted into the culture medium was analysed by SDS–PAGE and immunoblotting. As shown in Figure 3a, the upper band disappeared in the presence of tunicamycin (lane 3). These findings indicated that the upper and lower bands are glycosylated and non-glycosylated α-amylase, respectively, as seen in the *S. cerevisiae* secretion system (Tokunaga *et al.*, 1987, 1992).

To determine whether or not both glycosylated and non-glycosylated α-amylase molecules are enzymatically active, we actively stained α-amylase activity on non-denaturing PAGE. Figure 3b, lanes 1 and 2, shows that both glycosylated and non-glycosylated α-amylases were enzymatically active. The upper band was glycosylated α-amylase and the lower was non-glycosylated on non-denatured PAGE, since the former disappeared in the presence of tunicamycin (lane 1). We are the first to demonstrate that both glycosylated and non-glycosylated recombinant mouse salivary α-amylases are enzymatically active. In a control experiment, authentic human α-amylase (Sigma IX-A) migrated much faster than recombinant mouse α-amylases in this non-denaturing gel system. The upper glycosylated band that was bound to concanavalin A–Sepharose (data not shown) and the lower non-glycosylated band were both enzymatically active (lane 3).

Effects of carbon sources on the efficiency of α-amylase production

One advantage of *K. lactis* compared with *S. cerevisiae* is that it can utilize a wide variety of carbon sources (Wésolowski-Louvel *et al.*, 1996). For example, *K. lactis* assimilates lactose, L-sorbose, and L-lysine as carbon sources, but *S. cerevisiae* does not. We examined the effect of carbon sources on α-amylase secretion. Various carbon sources were added to YP medium, then we examined the cell growth of MD2/1 (pMI5) and secretion of α-amylase (Figure 4).

When glucose, lactose or galactose was used as a carbon source, the enzyme was efficiently secreted. When glycerol, ethanol or lysine was the carbon

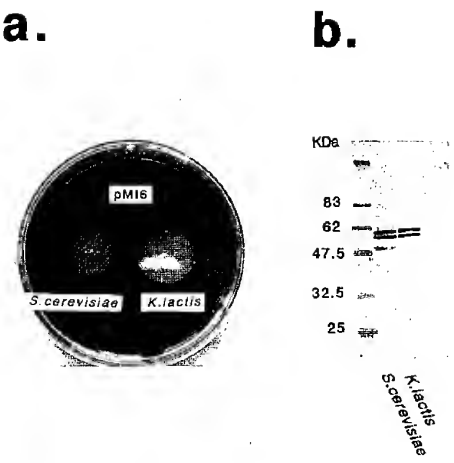

Figure 5. Comparison of the efficiency of α-amylase secretion between *K. lactis* and *S. cerevisiae* using the *K. lactis*/*S. cerevisiae* shuttle secretion vector, pMI6. (a) Halo assay of α-amylase secretion; *S. cerevisiae* YNN27 (pMI6) and *K. lactis* MD2/1(pMI6) are shown. (b) Immunoblots of the same samples. Trichloroacetic acid precipitates of culture medium (1·5 ml) were analysed.

source, the secretion gradually decreased, and the levels were very low in the presence of L-sorbose.

Direct comparison of secretion efficiency of α-amylase from K. lactis *and* S. cerevisiae *using the* K. lactis/S. cerevisiae *shuttle secretion vector pMI6*

The *K. lactis*/*S. cerevisiae* shuttle vector, pSK1, has replication origins from *K. lactis* (S11 fragment of pKD1) and *S. cerevisiae* (*ori* of 2 μm) plasmids. This shuttle plasmid was originally constructed by Prior *et al.* (1993) to characterize *K. lactis* homologous gene complementing an *S. cerevisiae* mutation and *vice versa*. Here, we constructed the shuttle secretion vector, pMI6, by inserting a mouse α-amylase secretion cassette encoded by the 3·2 kb *Sal*I-*Bam*HI fragment of pMI5 (*PGK* promoter-signal sequence of the 128 kDa killer precursor-α-amylase-*PGK* terminator) into *Sal*I/*Bam*HI-digested pSK1. Two strains of *K. lactis*, MD2/1 and PM6-7A, and three strains of *S. cerevisiae*, YNN27, S150-2b and W303-1B, were transformed with pMI6 in such a manner that the efficiency of α-amylase secretion from *K. lactis* and *S. cerevisiae* cells can be directly compared using the same secretion vector. These strains have been used often to study the secretion of heterologous proteins (Tokunaga *et al.*, 1990; Wésolowski-Louvel *et al.*, 1996).

Table 2. α-Amylase activities secreted from *K. lactis* and *S. cerevisiae* cells harboring *K. lactis*/*S. cerevisiae* shuttle secretion vector pMI6.

Strains	α-Amylase activity (μmol/min per ml medium)	Final cell density (Klett value)
K. lactis		
MD2/1	0·527	560
PM6-7A	0·148	630
S. cerevisiae		
YNN27	0·141	550
S150-2B	0·006	455
W303-1B	0·009	540

Transformants were initially cultured in SC medium and 0·5 ml of this seed culture was inoculated onto 5 ml of YP-2% galactose medium. After 72 h, activity secreted into the medium was assayed.

As shown in Figure 5a, both transformants formed clear halos, indicating that this shuttle vector functioned in both strains. *K. lactis* MD2/1 (pMI6) formed a larger halo than that of *S. cerevisiae* YNN27(pMI6). The immunoblotting profile was also the same, as shown in Figure 5b. The α-amylase activities in the culture supernatants assayed as described by Somogyi and Nelson revealed that *K. lactis* MD2/1(pMI6) secretes about four-fold more α-amylase activity than *S. cerevisiae* YNN27(pMI6) (Table 2). *K. lactis* PM6-7A(pMI6), which secretes less than MD2/1, secreted almost the same amount of α-amylase as *S. cerevisiae* YNN27(pMI6). Other *S. cerevisiae* strains harboring pMI6 secreted much less α-amylase; we could not resolve the significant difference in the secretion amounts among *S. cerevisiae* strains. These data indicated that *K. lactis* is more suitable as a host than *S. cerevisiae* with respect to the secretion of recombinant mouse α-amylase.

We measured the plasmid stability of MD2/1 (pMI6) and YNN27(pMI6) under the same conditions as the α-amylase assay described above. After 72 h of culture in rich medium without selective pressure, the stability of pMI6 was 11·4% in *K. lactis* MD2/1 cells, and 22·0% in *S. cerevisiae* YNN27 cells (average value in four experiments). *K. lactis* still secreted much more α-amylase regardless of its lower capacity for plasmid distribution than *S. cerevisiae*. This indicated that the increased plasmid stability in *K. lactis* cells will much improve the secretion efficiency.

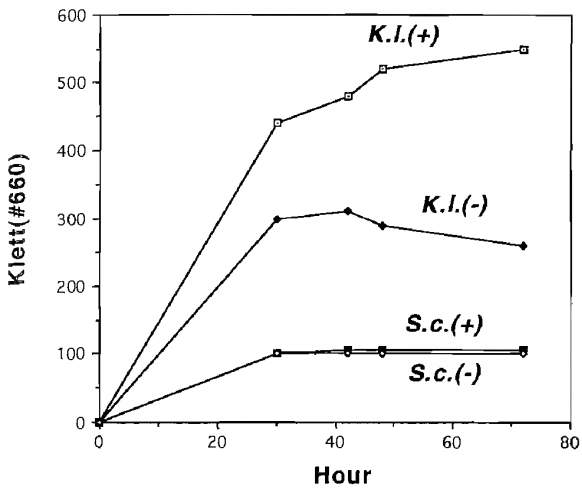

Figure 6. Cell growth of yeast transformants in the presence of starch. *K.l.*, *K. lactis* MD2/1(pMI6); *S.c.*, *S. cerevisiae* YNN27(pMI6); (+), YP medium containing 2% soluble starch; (−), YP medium without starch.

Assimilation of starch by K. lactis *transformants*

To determine whether or not *K. lactis* transformant cells secreting α-amylase can utilize starch as a carbon source for growth, *K. lactis* MD2/1(pMI6) and *S. cerevisiae* YNN27(pMI6) were cultured in YP medium with and without 2% soluble starch. As shown in Figure 6, the amount of *K. lactis* growth in YP medium with 2% starch was about two-fold higher than that without starch. The growth level of *K. lactis* transformants with starch was similar to that in YPD medium. The growth of *S. cerevisiae*, which was one-third that of *K. lactis* without starch, did not differ in YP medium with or without 2% starch. These data indicated that although both *K. lactis* and *S. cerevisiae* cells partly utilized the ingredients of YP medium as carbon sources, the former can efficiently assimilate oligosaccharides derived from starch digested by secreted α-amylase and can thus support their own growth. In contrast, *S. cerevisiae* cells harboring mouse α-amylase could not utilize oligosaccharides derived from starch as the carbon source (Figure 6). It is also conceivable that the total amount of α-amylase production from *S. cerevisiae* cells was lower than that from *K. lactis* cells under growth conditions using YP or YP-starch as the carbon source.

In conclusion, we induced the secretion of mouse α-amylase into the culture medium of *K. lactis* transformants using the pGKL 128 kDa killer secretion signal, and showed that *K. lactis* is superior to *S. cerevisiae* and *Sz. pombe* (Tokunaga *et al.*, 1993) for producing recombinant mouse α-amylase.

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ORIGINAL PAPER

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Allele-specific expression of the Mgi⁻ phenotype on disruption of the F_1-ATPase delta-subunit gene in *Kluyveromyces lactis*

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Abstract *Kluyveromyces lactis* is a petite-negative yeast that does not form viable mitochondrial genome-deletion mutants (petites) when treated with DNA-targeting drugs. Loss of mtDNA is lethal for this yeast but mutations at three loci termed *MGI*, for mitochondrial genome integrity, can suppress this lethality. The three loci encode the α-, β- and γ-subunits of mitochondrial F_1-ATPase. In this study we report the isolation and characterization of the *KlATPδ* gene encoding the δ-subunit of F_1-ATPase. The deduced protein contains 158 amino acids showing 72% identity to the protein from *Saccharomyces cerevisiae* and a putative mitochondrial targeting sequence of 23 amino acids. Disruption of the gene causes cells to become respiratory deficient while the introduction of *ATPδ* from *S. cerevisiae* restores growth on glycerol. Cells with a disrupted *ATPδ* gene, like strains with disruptions of α-, β- and γ-F_1-subunits, do not produce petite mutants when treated with ethidium bromide. However, unlike strains with disruptions in the three largest F_1-subunits, disruption of *ATPδ* in the presence of some *mgi* alleles does not abolish the Mgi⁻ phenotype. By contrast, elimination of *ATPδ* in other *mgi* strains removes resistance to ethidium bromide and ρ^0 mutants are not formed. Hence the *ATPδ* subunit of F_1-ATPase, while not mandatory for a Mgi⁻ phenotype, aids some *mgi* alleles in suppressing ρ^0 lethality.

Key words *Kluyveromyces lactis* · Mitochondrial genome integrity · F_1-ATPase δ-subunit gene · *ATPδ*-disruption

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Introduction

The energy transducing F_1 F_0-ATP synthases of bacteria, chloroplasts and mitochondria use a proton gradient, generated across a membrane by respiration or photosynthesis, to synthesize ATP (Futai et al. 1989; Senior 1990; Cox et al. 1992; Hatefi 1993; Boyer 1993; Deckers-Hebestreit and Altendorf 1996). In mitochondria, the F_0 component, containing 8–11 different subunits, spans the inner membrane, while the F_1 complex, composed of five proteins with the stoichiometry 3α, 3β, 1γ, 1δ, 1ε, extends into the matrix (Attardi and Schatz 1988; Grivell 1989; Poyton and McEwen 1996). For mitochondrial ATP-synthase, genes for the five F_1 subunits are encoded by nuclear DNA whereas genes for either two or three of the 8–11 subunits of the F_0 complex are located in mitochondrial DNA (mtDNA) (Poyton and McEwen 1996). Although the F_1 component of bacteria, chloroplasts and mitochondria all contain five separate subunits, only those of bacteria and chloroplasts share the same nomenclature and sequence homology (Cox et al. 1992). Differences between mitochondria and bacteria are found in the two smallest F_1 subunits. In bacteria the ε-subunit shares sequence homology with the δ-subunit of mitochondria while the ε-subunit of mitochondria does not have an equivalent protein in bacteria.

A better understanding of the role played by the three F_1 subunits in the operation of ATP synthase has been provided by structural analysis of bovine mitochondrial F_1 (Abrahams et al. 1994). The α- and β-subunits alternate in a hexameric complex where the catalytic site for ATP synthesis occurs in β-subunits at a contact region with α-subunits. A central space formed by the hexameric array is occupied by amino- and carboxy terminal α-helices of the γ-subunit. Apart from another small portion of the γ-subunit, the central part of this protein, together with the δ- and ε-subunits, have not been located. One view is that these proteins form part of a stalk that helps F_1 attach to F_0 in the membrane (Walker and Collinson 1994; Deckers-Hebestreit and Altendorf 1996).

Table 1 Genotype and source of yeast strains

Strain	Relevant genotype	Source or reference
CK56-7A	*α, ade1, lysA1, uraA1, mgi1*-1	Chen and Clark-Walker (1996)
CK99-7C	*α, his7, metA1, trpX, uraA1, mgi2*-1, *mex1*-1	Chen and Clark-Walker (1995)
CK103-1B	*α, ade1, adeT*-600, *lysA1, uraA1, mgi1*-9	Clark-Walker et al., in preparation
CK113/1	*α, ade1, uraA1, mgi2*-6	Clark-Walker et al., in preparation
CK359/1	*α, ade1, lysA1, uraA1, mgi1*-1, *atpδ::URA3*	This study
CK361/2	*α, his7, metA1, trpX, uraA1, mgi2*-1, *mex1*-1, *atpδ::URA3*	This study
CK362/1	*α, ade1, uraA1, mgi2*-6, *atpδ::URA3*	This study
CK363/1	*a, metA1, uraA1, mgi5*-2, *atpδ::URA3*	This study
CK364/1	*a, ade1, ade2, lysA1, uraA1, mgi1*-9 *atpδ::URA3*	This study
CW26-1A	*a, metA1, uraA1, mgi5*-2	Clark-Walker et al., in preparation
PM6-7A	*a, ade2, uraA1*	Chen *et al.* (1992)
PM67A/ *atpδ::URA3*	*a, ade2, uraA1, atpδ::URA3*	This study

Evidence from parallel studies with *Escherichia coli* and spinach chloroplasts supports the view that the γ-subunit rotates relative to the hexameric ring of the α- and β-subunits (Duncan et al. 1995; Sabbert et al. 1996; Noji et al. 1997) and that in *E. coli* the ε-subunit (equivalent to the δ-subunit in mitochondria) shows relative movement during ATP hydrolysis (Aggeler and Capaldi 1996).

Our interest in the yeast mitochondrial δ-subunit has arisen from investigations into a phenomenon seemingly unrelated to ATP synthesis. A minority of yeasts, exemplified by *Saccharomyces cerevisiae*, readily form respiratory deficient (petite) mutants that lose mtDNA when treated with DNA targeting drugs (Ephrussi 1953; Bulder 1964 a, b; De Deken 1966; Slonimski et al. 1968; Clark-Walker et al. 1981). By contrast, *Kluyveromyces lactis*, as a representative of most yeasts, does not form viable petite-negative mutants upon exposure to such drugs (Bulder 1964 a b; Heritage and Whittaker 1977). Indeed, elimination of mtDNA is lethal for wild-type *K. lactis* (Clark-Walker and Chen 1996). However, it has been discovered using this yeast that a class of nuclear genome mutants termed *mgi*, for *m*itochondrial *g*enome *i*ntegrity, can suppress the petite-negative phenotype so that mtDNA deletion mutants can be recovered (Chen and Clark-Walker 1993). Characterization of three *MGI* loci has shown that they encode the α-, β- and γ-subunits of F_1-ATPase (Chen and Clark-Walker 1995, 1996). Specific mutations, producing amino-acid substitutions, together with an assembled F_1 particle, appear to be necessary for a Mgi^- phenotype as disruption of any of these three genes results in the loss of suppressor activity.

As a foundation for the possible identification of further *MGI* loci, and to gain an understanding of how *mgi* mutations permit the loss of mtDNA, we undertook to isolate and characterize the *ATPδ* gene encoding the F_1 δ-subunit of *K. lactis*, and to examine any possible involvement of this subunit in the conversion between petite-negative and -positive phenotypes.

Materials and methods

Strains and media. The *K. lactis* strains used in this study are listed in Table 1. The isolation and characterization of three new *mgi* alleles *mgi1*-9, 2-6 and 5-2, not previously described, will be reported in a subsequent publication (Clark-Walker et al., in preparation). *S. cerevisiae* AH22 *Mata, leu2*-3, 112, *his4* was used as a source of genomic DNA for PCR amplification. Complete medium (GYP) contains 0.5% Bacto yeast extract, 1% Bacto Peptone, and 2% glucose. Glycerol medium (GlyYP) contains 2% glycerol in place of glucose. Minimal medium (GMM) contains 0.67% Difco yeast nitrogen base without amino acids and 2% glucose. Nutrients essential for auxotrophic strains were added at 25 μg/ml for bases and 50 μg/ml for amino acids. Ethidium bromide (EB) medium is GYP plus EB at 16 μg/ml.

Amplification by the polymerase chain reaction. Amplification of *ATPδ* from *S. cerevisiae* was carried out as described by Mullis and Faloona (1987) using a *Taq* DNA polymerase kit supplied by Promega. The forward primer, 5′(+134)GTGGCTCCGAAGTTACTC(+151)3′, and reverse primer, 5′(449)TCTACTTGAATTGCAGC(433)3′, were designed from the *S. cerevisiae ATPδ* sequence (Giraud and Velours 1994) and incorporated into a reaction with 50 ng of DNA from AH22 (Chen and Clark-Walker 1995). The purified PCR product had an electrophoretic mobility equivalent to 316-bp of the expected fragment.

Isolation and sequencing of K. lactis ATPδ. The PCR-amplified fragment of *ATPδ* was labelled with [^{32}P] dATP by the random primer method and used to probe a *K. lactis* partial *Sau*3AI gene bank cloned in the *K. lactis*/*E. coli* shuttle vector KEp6 (Weslowski-Louvel et al. 1986). The two positive colonies were subjected to further analysis and one, containing a plasmid with a 5-kb insert, was mapped using restriction endonucleases. For nucleotide-sequence determination, appropriate DNA fragments were cloned into the sequencing vectors pTZ18 U and pTZ19 U (Pharmacia) and single-stranded templates were subsequently obtained for sequence determination using the dideoxy chain-termination procedure (Sanger et al. 1977). In addition to the 'universal primer' a synthetic oligonucleotide, 5′(+287)CTGTCCAACCAGACT(+301)3′, was prepared to complete the sequence.

Disruption of ATPδ. Gene disruption was carried out by the one-step replacement procedure (Rothstein 1983). A plasmid containing a 1.65-kb *Stu*I-*Bam*HI fragment (see Fig. 1), was opened at the unique *Bgl*II site within the *ATPδ* gene and a 1.1-kb *Bgl*II fragment containing the *URA3* gene of *S. cerevisiae* was inserted. Disruption of *ATPδ*

in *K. lactis* PM6-7A and various *mgi* strains was achieved by transformation with a 1.74-kb *Msc*I-*Bsp*HI fragment obtained from the above construct. Transformants were selected for Ura$^+$ and the correct gene replacement was verified by digestion of genomic DNA with *Msc*I-*Bsp*HI and hybridization to [^{32}P]-labelled *ATPδ*.

Complementation of K. lactis containing a disrupted ATPδ. The plasmid pCXJ22-ScATPδ was constructed by cloning a 2.6-kb *Bam*HI-*Eco*RI fragment, containing *ScATPδ* isolated from pMFG4 (Giraud and Velours 1994), into the *Bam*HI and *Eco*RI sites of the *K. lactis* vector pCXJ22 (Chen 1996). The *KlATPδ*-disrupted strain PM6-7A/ATPδ::URA3 was transformed with pCXJ22-*ScATPδ*. Transformed cells were plated on GMM plates, grown at 28°C for 24 h, and then replica-plated to GlyYP medium. Respiratory competent colonies (Gly$^+$) were obtained, indicating that Sc*ATPδ* can complement the disrupted allele of *KlATPδ*.

Viability determination. Cells were grown in GYP medium to stationary phase and spread onto the surface of a GYP agar slice. Individual cells were removed with a de Fonbrun micromanipulator to fresh medium to allow the development of individual colonies.

Nucleotide-sequence accession number. GenBank U88046. The sequence of the *K. lactis* F_1-ATPase δ-subunit gene, *KlATPδ* has been given the GenBank accession number U88046.

Results and discussion

Cloning and sequencing of *ATPδ*

A *K. lactis* gene bank was probed with a fragment of the δ-subunit gene from *S. cerevisiae*. Of the two positive clones identified, one contained a plasmid with an insert of 5 kb that hybridized strongly with the probe. A map of restriction endonuclease sites was constructed and suitable fragments were cloned and sequenced (Fig. 1A). The nucleotide sequence of *ATPδ* and flanking regions, together with the deduced amino-acid sequence, is illustrated in Fig. 2. An open reading frame of 158 codons is present that has 70% nucleotide, and 72% amino-acid, identity to the *ATPδ* gene from *S. cerevisiae* (Fig. 3; Giraud and Velours 1994). Of note is the absence of a codon in the middle of the gene which in *S. cerevisiae* specifies asparagine. The amino-terminus of the deduced protein contains basic and hydrophobic amino acids that are elements of a mitochondrial targeting signal. In *S. cerevisiae* the mature δ-subunit begins with alanine which is preceded by codons specifying a maturation site (R-X-Y). Likewise, these sequence elements are conserved in the δ-subunit of *K. lactis* suggesting that a similar mature protein is likely to be produced (Fig. 3).

Disruption of *ATPδ*

To disrupt the *ATPδ* gene, a plasmid was constructed containing the *URA3* gene of *S. cerevisiae* inserted at a *Bgl*II site within the coding region (Fig. 1 A). A fragment containing the disrupted *ATPδ* gene, produced by digestion with *Msc*I-*Bsp*HI, was then used to transform the *K. lactis* strain PM6-7A. Transformants were selected on uracil-free

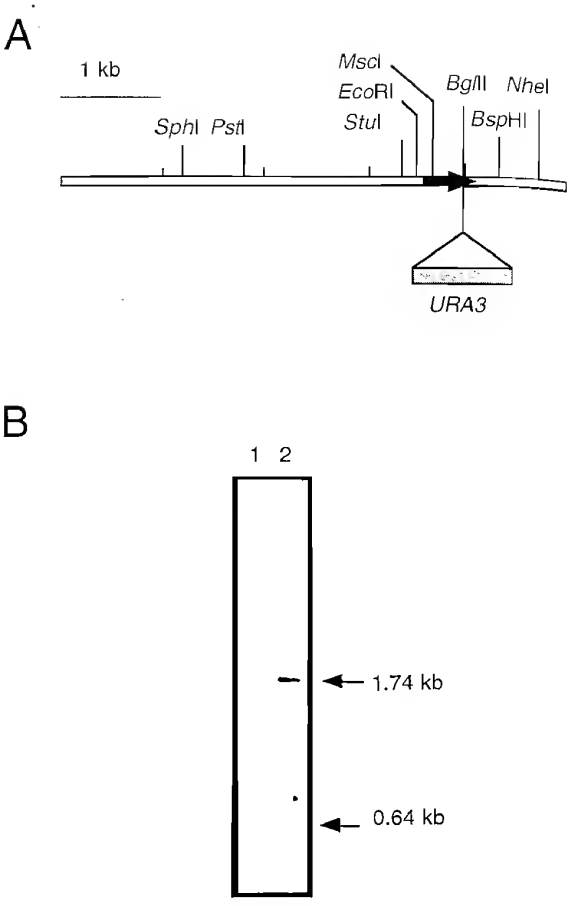

Fig. 1 **A** Physical map of a 5-kb fragment of *K. lactis* genomic DNA containing the *ATPδ* gene. The position of the gene and the direction of transcription is indicated by the *black arrow*. For gene disruption, a 1.1-kb DNA segment, containing the *S. cerevisiae URA3* gene, has been inserted at the *Bgl*II site. **B** Southern-blot analysis of gene disruption. Genomic DNA from the parental strain, PM6-7A, *lane 1*, and the transformant, *lane 2*, have been digested with *Msc*I and *Bsp*HI and hybridized with [^{32}P]-labelled *ATPδ*

medium and examined for replacement of the resident *ATPδ* gene by gel electrophoresis and Southern-blot analysis. As illustrated in Fig. 1B, a Ura$^+$ transformant has a DNA fragment, hybridizing to the *ATPδ* probe, that has increased in size by 1.1 kb due to the insertion of *URA3*.

Strains containing a disrupted *ATPδ* do not grow on glycerol and show a highly sectored colony morphology on glucose (data not shown). Sectoring is probably a consequence of low viability. Micro-manipulation of individual cells shows that only 3% of cells in a liquid culture at the end of exponential growth form colonies whereas 68% of cells in a culture of the parent, PM6-7A, form colonies. Viable cells still contain mtDNA since transformation with a plasmid containing the *ATPδ* gene from *S. cerevisiae* leads to the restoration of growth on glycerol; however, transformants do not show resistance to EB. Complementation by the *S. cerevisiae* gene confirms that *ATPδ* of *K. lactis* encodes the F_1 δ-subunit. The reciprocal experiment was not attempted because *S. cerevisiae* strains with a dis-

Fig. 2 Nucleotide sequence of the *K. lactis ATPδ* gene and deduced amino-acid sequence. Key restriction endonuclease sites used in gene disruption have been included

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                                                                  GATTTTTG -235
AAAATGAAAAGAATGGGATTTTAGTTTTAATTACAGCTTTACGATATTTGTGAATAAGGTTTACTAGAACTGTTCTTG -157
                                                                      EcoRI
TTGGACTACTGTTGTTCCTTCTTAGACTTTTGTATTTTGATCTTTGTTTAGTGTTATTAGATTTTGTTTCAATTGAAT  -79
TCATTACAGTCTGTTTTAATTGCTTCTTGTCCAGAATCGAAATCATAAGAACACATTACCAGAAAATAATCACCTATA   -1

                                  MscI
ATGTTCCGTTTATCTGCTGCTAGAACTTTGGCCAAATCTGTCAACACCGTTGTTGCTAAGCGTACTTATGCTGAAGCT   78
 M  F  R  L  S  A  A  R  T  L  A  K  S  V  N  T  V  V  A  K  R  T  Y  A  E  A    26

GCCGATGGTGCTTTGAAGTTGCAATTTGCTTTGCCACATCAAACCTTGTTTTCTGGTACTCCAGTTACCCAAGTCAAC  156
 A  D  G  A  L  K  L  Q  F  A  L  P  H  Q  T  L  F  S  G  T  P  V  T  Q  V  N    52

TTGCCAGCTAAGTCTGGTCAAATCGGTATCTTGGCTAACCACGTTCCAACTGTTGAACAATTGGTTCCAGGTGTTGTT  234
 L  P  A  K  S  G  Q  I  G  I  L  A  N  H  V  P  T  V  E  Q  L  V  P  G  V  V    78

GAAGTCTTGGAAGGTTCTTCTAAGAAGTTCTTTGTTTCCGGTGGTTTCGCTACTGTCCAACCAGACTCTACCTTGGCT  312
 E  V  L  E  G  S  S  K  K  F  F  V  S  G  G  F  A  T  V  Q  P  D  S  T  L  A   104

                                                  BglII
ATCACTTCCGTTGAAGCATTCCCATTAGAGTCTTTCTCTCCAGAAAACGTTAGATCTCTGTTGGCTGAAGCTCAAAAG  390
 I  T  S  V  E  A  F  P  L  E  S  F  S  P  E  N  V  R  S  L  L  A  E  A  Q  K   130

AACGTCTCCTCTGCTGACGAAGTTGCTGCTGCCGAAGCTGCTATCCAACTTGAAGTTTTGGAAGCTTTGCAAGCCGCT  468
 N  V  S  S  A  D  E  V  A  A  A  E  A  A  I  Q  L  E  V  L  E  A  L  Q  A  A   156

TTGAAATAATTGAGATGAACGAAGAATTACAATTGGACCAAGGTATAATACACTTGATACGGATACTCTACTTGGAAG  546
 L  K  *                                                                          158

CATTGTTTTCCCGGTTGATTGTAAACCTGTCTCGGCGTTTGTATTATCCTTCGTCTGCTTTTTTTTCATTATTCTAAT  624

                                                  BspHI
TCCTATTATTCCATTGGCACAAGCTTACACAACACAACGACGAATACATTTCATGATTTAGCCTAAAGACCCTTTTTT  702
GTTGCTCCGGTCATTGTCCTTTCTTTCCATACAATACTGTAGTGTGAACGT                              753
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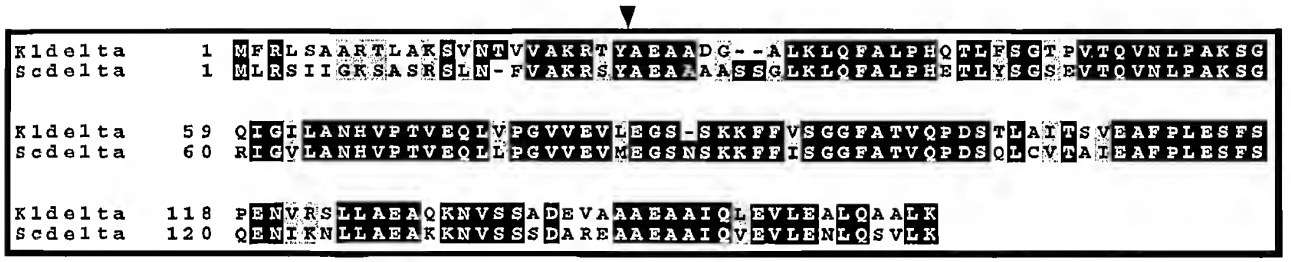

Fig. 3 Alignment of F_1 δ-subunit sequences from *K. lactis* (158 amino acids) and *S. cerevisiae* (160 amino acids). *Black shading* indicates identity and *grey* represents conserved amino acids. The two proteins have 114 identical amino acids. An *arrow* indicates the cleavage site of the *S. cerevisiae* precursor protein

rupted *ATPδ* do not possess functional mtDNA due to their total conversion to petites (Giraud and Velours 1994). In view of this observation with *S. cerevisiae* it is likely that low viability and colony sectoring on disruption of *ATPδ* in *K. lactis* is due to instability of mtDNA leading to the formation of inviable petite mutants.

A similar colony sectoring phenotype has been found on disruption of the *MRF1* gene encoding a mitochondrial peptide release factor in *K. lactis* (Pel et al. 1996). Low viability of *MRF1* disruptants is viewed as arising from the formation of cytoplasmic petites that do not survive. In *S. cerevisiae*, inactivation of *MRF1* leads to excessive production of petite mutants (Pel et al. 1992), which accords with the observation that interference in mitochondrial translation results in the de-stabilization of mtDNA (Myers et al. 1995).

Disruption of *ATPδ* in *K. lactis* does not alter the Mgi^+ phenotype since strains do not grow in the presence of 16 μg/ml of EB (Fig. 4). In this respect, disruption of *ATPδ* produces the same Mgi^+ phenotype as null mutants of *MGI1, 2* and *5* encoding the α-, β- and γ-subunits of F_1 (Chen and Clark-Walker 1995, 1996). However a notable difference between disruptants of *ATPδ* and the three other loci is observed in the presence of a *mgi* allele.

Disruption of *ATPδ* in different *mgi* strains

Previous studies have shown that disruption of any of the three *MGI* genes in *mgi* mutants abolishes the Mgi^- phenotype. Such strains are no longer resistant to EB at 16 μg/ml, nor are petite mutants produced. However disruption of *ATPδ* in *mgi* mutants is not so simple. Strains containing different *mgi* alleles (Table 1) respond differently on disruption of *ATPδ*. Transformants of the various *mgi* mutant strains were checked for replacement of the resident *ATPδ* by Southern blotting and hybridization (data not shown) and examined for growth on glycerol and their

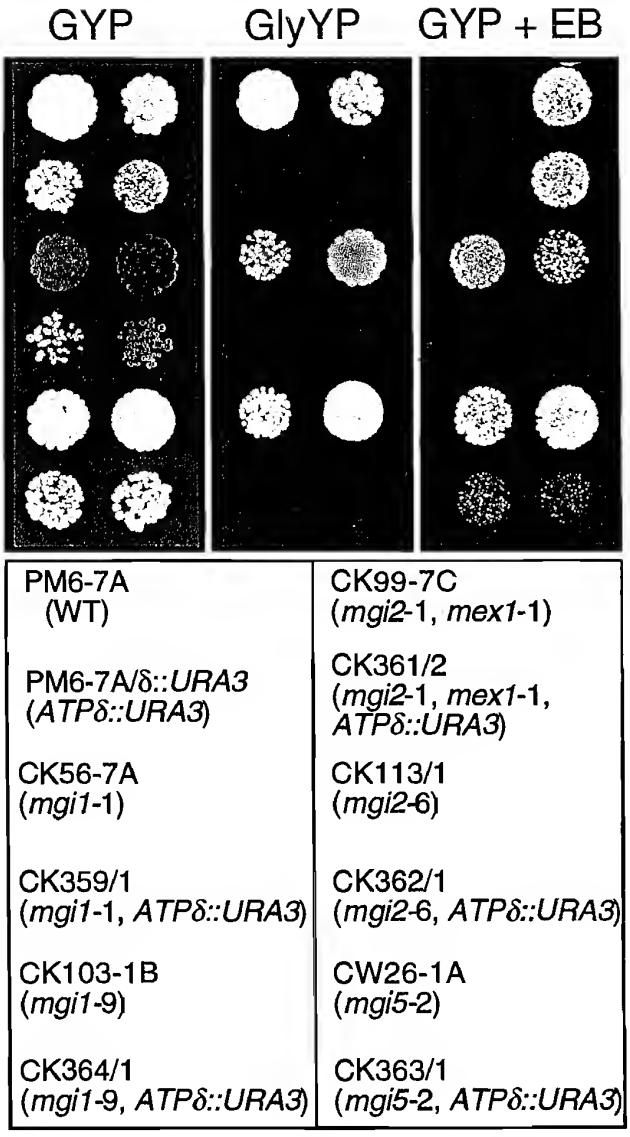

Fig. 4 Growth of *K. lactis* strains on glucose (*GYP*), glycerol (*GlyYP*) and response to ethidium bromide at 16 μg/ml. Cells were grown to stationary phase, diluted to approximately 10^4 cells/ml and 10-μl aliquots applied to plates which were incubated at 28°C for 5 days (GYP and GlyYP) and 7 days for GYP plus EB. Genotypes of strains are listed in Table 1

response to EB (Fig. 4). All *mgi* strains, which were chosen because of their Gly^+ phenotype, fail to grow on glycerol after disruption of *ATPδ*, in accord with the requirement of this subunit for oxidative phosphorylation. However, growth does occur on EB with *ATPδ*-disrupted strains containing *mgi2-1*, *mex1-1* and to a lesser extent with *mgi1-9* and *mgi5-2*, whereas a Mgi^- phenotype is not present in disrupted strains with *mgi1-1* and *mgi2-6* mutations.

While these results indicate that the Atpδ subunit is not absolutely required for a Mgi^- phenotype, there is a clear need for this protein in some *mgi* mutants. In this respect it is noteworthy that assembly of a core F_1 particle, composed of α-, β- and γ-subunits, can still occur in *Escherichia coli* in the absence of the ε-subunit (equivalent to δ in yeasts) although energy linked functions are abolished (Klionsky and Simoni 1985). Hence, by analogy with *E. coli*, a core F_1 particle, lacking the δ-subunit, may form in the mitochondria of some *mgi* mutants and be sufficient for suppression of ρ^0 lethality. Alternatively, in strains with different *mgi* alleles, assembly or stablilization of a core F_1 particle could be dependent on the presence of Atpδ.

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Enolase and Glycolytic Flux Play a Role in the Regulation of the Glucose Permease Gene *RAG1* of *Kluyveromyces lactis*

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ABSTRACT

We isolated a mutant, *rag17*, which is impaired in glucose induction of expression of the major glucose transporter gene *RAG1*. The *RAG17* gene encodes a protein 87% identical to *S. cerevisiae* enolases (Eno1 and Eno2). The *Kleno* null mutant showed no detectable enolase enzymatic activity and has severe growth defects on glucose and gluconeogenic carbon sources, indicating that *K. lactis* has a single enolase gene. In addition to *RAG1*, the transcription of several glycolytic genes was also strongly reduced in the Δ*Kleno* mutant. Moreover, the defect in *RAG1* expression was observed in other mutants of the glycolytic pathway (hexokinase and phosphoglycerate kinase). Therefore, it seems that the enolase and a functional glycolytic flux are necessary for induction of expression of the Rag1 glucose permease in *K. lactis*.

IN most strains of *Kluyveromyces lactis*, the glucose uptake system relies on two nonredundant glucose transporters: a low-affinity permease encoded by *RAG1* (Wésolowski-Louvel *et al.* 1992a) and a high-affinity permease encoded by *HGT1* (Billard *et al.* 1996). *HGT1* is constitutively expressed (Billard *et al.* 1996); expression of *RAG1* is activated in the presence of high concentrations of glucose (Chen *et al.* 1992; Wésolowski-Louvel *et al.* 1992a). The Rag1 permease is necessary for supporting fermentative growth, which requires a high flow of substrate. In the absence of Rag1, the cell becomes respiration dependent for growth on high-glucose media. Thus, *rag1* cells have the Rag$^-$ phenotype: they cannot grow on 5% glucose in the presence of antimycin A, which blocks respiration (Goffrini *et al.* 1989; Wésolowski-Louvel *et al.* 1992b).

To date, studies of Rag$^-$ mutants have identified three key components that are involved in the positive regulation of *RAG1* expression: the glucose sensor Rag4 (Betina *et al.* 2001), hexokinase Rag5 (Prior *et al.* 1993), and casein kinase I Rag8 (Blaisonneau *et al.* 1997).

In this report we present the characterization of another gene in *K. lactis* implicated in *RAG1* regulation: *RAG17* (*KlENO*) coding for enolase.

Sequence data from this article have been deposited with the EMBL Data Library under accession no. AJ586240.

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MATERIALS AND METHODS

Yeast strains and growth conditions: Yeast strains are described in Table 1. Yeast cells were grown at 28° in a complete YP medium containing 1% Bacto yeast extract, 1% Bactopeptone (Difco, Detroit), supplemented with either 2% glucose (YPG) or a specified carbon source. Minimal medium containing 0.7% yeast nitrogen base without amino acids (Difco) and 2% glucose was supplemented with auxotrophic requirements. The Rag phenotype was tested on 5% complete glucose medium supplemented with 5 μM antimycin A. For G418 medium, YPG plates were supplemented with geneticin (200 μg/ml; Life Technologies). 5-FOA medium was prepared according to Boeke *et al.* (1984). The media for plates were solidified by the addition of 2% Bactoagar (Difco). *Escherichia coli* XL1-blue was used as a cloning host and DNA propagation and was grown in LB medium.

Genetics methods have been described previously (Wésolowski *et al.* 1982; Goffrini *et al.* 1989).

Yeast transformation: Replicative transformation of *K. lactis* was performed by electroporation. For integrative transformation of *K. lactis*, the procedure described by Dohmen *et al.* (1991) was followed. Replicative and integrative transformations of *Saccharomyces cerevisiae* were standard.

Construction of deletion strains: One-step gene deletions using *kanMX4* or *HIS3* selection markers bearing PCR-generated long flanking homology (LFH-PCR; Wach 1996) was used to construct the mutant strains MWK3, MLK43, MLY702, and MLY703 (Table1). The primers used for the *KlENO* LFH-PCR synthesis were P5′ *KlENO* (5′-CACGTTCACAATCCAGGC ACC-3′), P5′L *KlENO* (5′-**CCGTCGACCTGCAGCGTACGTGG** CATGTTTTTTTG-3′), P3′L *KlENO* (5′-**GCTCGAATTCATC GATGAT**ATTTGACTGTCACCAAC-3′), and P3′*KlENO* (5′-AGC GAAGATAGCGTTGGAACC-3′). The primers used for the *KlPGK* LFH-PCR synthesis were P5′ *KlPGK* (5′-ACGATCTCGTCCTAG TGGAAGC-3′), P5′L *KlPGK* (5′-**GGGGATCCGTCGACCTGC AGCGTACG**CATTTTTATTAATTCTTGATCG-3′), P3′L *KlPGK* (5′-**CGAGCTCGAATTCATCGATGAT**ATAAATGTAGGATC CATCATCCC-3′), and P3′*KlPGK* (5′-TACGATGAACCAGTGCA CAAG-3′). The primers used for the *ScENO2* LFH-PCR synthesis

were P5′ *ScENO2* (5′-ATCCTACTCTTGCCGTTGCCATCC-3′), P5′L *ScENO2* (5′-**GGGGATCCGTCGACCTGCAGCGTACGCA** TTATTATTGTAT GTTATAGTA-3′), P3′L *ScENO2* (5′-**AAACGA GCTCGAATTCATCGATGATA**TAAAGTGCTTTTAACTAAGA ATT-5′), and P3′ *ScENO2* (5′-GTACTCATAGAGGTAGGCTAGA CC-3′). For all the P5′L and P3′L primers, the *Kan*MX4 or *HIS3* MX6 regions are in boldface type. All correct integrations were verified by Southern blot or PCR.

In *S. cerevisiae*, the *ScENO2* gene was first disrupted in the diploid strain MLY701 by using a *kanMX4* or a *HIS3MX6* disruption cassette, yielding MLY702 and MLY703 strains, respectively (Table 1). After meiotic analysis of these two diploid strains, the Δ*Sceno2* haploid strains MLY704 (Δ*Sceno2::kanMX4*) and MLY708 (Δ*Sceno2::HIS3*) were obtained. The double null mutant strain Δ*Sceno1* Δ*Sceno2* was then constructed by crossing the MLY708 strain with the Δ*Sceno1* strain Y07286 (Table 1), yielding the MLY713 diploid.

In *K. lactis*, the *KlENO* and *KlPGK* genes were disrupted in the MW270-7B strain using the corresponding *kanMX4* disruption cassettes, yielding MWK3 and MLK43 strains, respectively.

Plasmid constructions: The *ENO2* gene of *S. cerevisiae* was first PCR amplified from MLY701 genomic DNA by using the Pfx polymerase (Invitrogen, San Diego) and the P5′ *ScENO2*/P3′ *ScENO2* primers. After phosphorylation with T4 polynucleotide kinase (New England Biolabs, Beverly, MA), the *ScENO2* PCR product was cloned into the *CEN-URA3* vector pRS416 (Sikorski and Hieter 1989) linearized with *Sma*I, yielding pML180. This plasmid could complement the slow growth of the *ScΔeno2* mutant (MLY708, Table 1) and thus contained a functional copy of *ScENO2*. The 2.9-kb *Xho*I-*Not*I fragment of pML180, containing *ScENO2*, was then cloned into the *CEN TRP1* vector pRS414 (Sikorski and Hieter 1989) digested with *Xho*I and *Not*I, yielding pML190. The *ScENO2* was also introduced into a *K. lactis* centromeric vector by cloning the *Xba*I-*Xho*I 2.9-kb fragment of pML180 between the *Xba*I and *Sal*I sites of the *CEN URA3* pCXJ18 vector (Chen 1996), yielding pML187.

The *K. lactis KlENO* gene was cloned into a *S. cerevisiae* vector by subcloning the 5.5-kb *Bam*HI fragment of pMW1 (see below) into the *Bam*HI site of pRS414, yielding pML183.

Cloning and sequencing of *RAG17/KlENO* gene: The *RAG17* gene was cloned by *in vivo* complementation of *rag17* mutation (strain MWK2) with a *K. lactis* genomic library made in the KCp491 vector (Prior *et al.* 1993). Of 5000 Ura$^+$ transformants, 4 were found to be Rag$^+$. The complementing plasmids extracted from these four transformants and amplified in *E. coli* showed that three of them carried the same plasmid, pMW1 containing a 7.5-kb insert (Figure 1), and one a plasmid with a 10-kb insert overlapping with a pMW1 insert.

A 2856-bp fragment containing the entire *RAG17/KlENO* gene was sequenced on both strands.

Preparation of yeast RNA and probes: Total RNA was extracted from cells grown to an OD_{600} of 2–3. Poly(A$^+$)-enriched mRNA was obtained using mRNA Separator (CLONTECH, Palo Alto, CA). Several gene probes used were restriction fragments: *RAG6* probe was a 1.3-kb *Eco*RI-*Sal*I fragment (Bianchi *et al.* 1996); *HGT1* probe was a 1.75-kb *Eco*RI-*Hin*dIII fragment containing the *HGT1* gene (Billard *et al.* 1996). Other probes were obtained by PCR amplification using either *K. lactis* genomic DNA or the cloned genes as template. The oligonucleotides used were: 0487 (5′-GGGGTCGTAGAATTGGT-3′) and 0369 (5′-GACGTAACCGTAGTAGAAG-3′) for *RAG1*; RAG2-up (5′-TGTACGTTGATGGTACCAACG-3′) and RAG2-down (5′-CAAG ATAGAACCAGTAGAGTA-3′) for *RAG2*; p2E1/9 (5′-GCCATCT GTGCAGCATCAAA-3′) and p2E1/5 (5′-GGGAAGAAGATCGAG TAGTG-3′) for *RAG4*; 470 (5′-GTGCCAGCTAATTTGATGGA-3′) and 471 (5′-AGCAGCCACCAATTGGATTG-3′) for *RAG5*; PENO1 (5′-ACTGGCTGTCTGACTAGC-3′) and EndoY (5′-GTC TTAGCACCGGCCAAGTC-3′) for *KlENO*; SCK1-up (5′-GAACAC CAACATGTTCGCTACTC-3′) and SCK1-down (5′-GACAACG AACGCAGTATCTTCGC-3′) for *SCK1*; GCR1-up (5′-CACCAG TAACATGATACGGTCC-3′) and GCR1-down (5′-GACCACCAT CAGATATACTGTTGCC-5′) for *KlGCR1*; GCR2-B (5′-TCAGC GATTTCAACAGATAT-3′) and GCR2-D (5′-CTCATTGATCTGT TCCATAG-3′) for *KlGCR2*. In all cases, specific probes of *K. lactis* actin gene (*KlACT*), *KlrRNA 18S* gene (*18S*), or *KlAAC* gene were used in parallel as quantitative references. In the case of *KlACT*, the probe was a 900-bp *Eco*RI-*Hin*dIII fragment. *18S* and *KlAAC* probes were amplified by PCR using the following oligonucleotides: PKl-18S f (5′-ATCCTGCCAGTAGTCATATGC-3′) and PKl-18S r (5′-CCACAAGGAGTACAGGTTAGC-3′) for *rRNA 18S*; P5′ KlAAC (5′-AGATGAAATGATCAAGCAAGG-3′) and P3′KlAAC (5′-CGTACATGGAGATAACACCGG-3′) for *KlAAC*.

Northern blot hybridization was quantified by scanning with a Cyclone Phosphoimager (Packard, Meriden, CT).

Preparation of cell-free extract and enzyme assays: Whole-yeast-cell extracts were prepared by glass-bead disruption of cells isolated from log-phase cultures grown in YP medium containing either 2% glucose or 2% glycerol. Enolase activity was assayed (Clifton *et al.* 1978) and normalized to protein concentration determined by the Bradford protein assay (Bio-Rad, Richmond, CA).

RESULTS

Isolation of the *KlENO* gene and deduced amino acid sequence of its product: In *K. lactis*, gene replacement by homologous recombination can be accomplished, but at lower frequencies compared to *S. cerevisiae*. Usually, the gene disruption cassette recombines at ectopic sites in the genome. While attempting to construct a *rag4::URA3* gene disruption (Betina *et al.* 2001), we identified a Rag$^-$ mutant that is not allelic to *rag4*, although *RAG1* transcription is highly reduced in this mutant. The mutation is also not allelic to *rag5* and *rag8* mutations, both of which affect genes that positively regulate the transcription of the *RAG1* gene (Chen *et al.* 1992). We named the mutation *rag17-1* (strain MWK1 in Table 1). The precise position of the *URA3* insertion in the gene is not known.

RAG17 was isolated from a CEN-based *K. lactis* plasmid library by complementation of the Rag$^-$ phenotype of the *rag17-1* mutation (materials and methods). The partial nucleotide sequence of the DNA fragment in the complementing plasmid (Figure 1 and materials and methods) revealed the presence of three ORFs: one encodes a protein 40% identical to the Fmo of *S. cerevisiae*, a flavin-containing monooxygenase involved in protein folding (Suh *et al.* 1999); another encodes a protein highly similar to the enolases of *S. cerevisiae*, Eno1 and Eno2 (Holland *et al.* 1981); the third encodes a protein 28% identical to Rax1 of *S. cerevisiae*, a protein implicated in bud site selection (Chen *et al.* 2000). Because of the Rag$^-$ phenotype of the mutation, the best candidate for *RAG17* was the ORF that encodes the glycolytic enzyme enolase (KlENO). The predicted protein (437 amino acids) is 88 and 87% identical to ScEno2

TABLE 1

Yeast strains

Strain	Relevant genotype	Reference
K. lactis		
MW270-7B	*MAT***a** *uraA1-1 metA1-1 leu2*	Billard *et al.* (1996)
PM4-4B	*MAT*α *uraA1-1 ade2-1*	Goffrini *et al.* (1991)
PM6-7A	*MAT***a** *uraA1-1 adeT-600*	Chen *et al.* (1992)
MWK1	Isogenic to MW270-7B except *Kleno::URA3 (rag17-1)*	This work
MWK2	Isogenic to MW270-7B except *Kleno::ura3*	This work
MWK3	Isogenic to MW270-7B except *KlenoΔ1::kanMX4*	This work
MW352-2D	*MAT*α *metA1-1 ade2-1 Kleno::URA3*	This work
MLK43	Isogenic to MW270-7B except *Klpgk Δ1::kanMX4*	This work
MWK11	Isogenic to MW270-7B except *rag5Δ2::URA3 (ΔKlhxk)*	This work
MWK12	Isogenic to PM4-4B except *rag2Δ1::URA3 (ΔKlpgi)*	Goffrini *et al.* (1991)
MWK13	Isogenic to PM6-7A except *rag6Δ1::URA3 (ΔKlpdc)*	Bianchi *et al.* (1996)
S. cerevisiae		
Y07286	*MAT***a** *ura3 Δ0 met15 Δ0 leu2 Δ0 his3Δ1 YGR254w*[a]*::kanMX4*	Euroscarf
BM64-1A	*MAT***a** *ura3-1 trp1Δ2 leu2-3,112 his3-11 ade2-1 can1-100*	Euroscarf
BM64-1B	*MAT*α *ura3-1 trp1Δ2 leu2-3,112 his3-11 ade2-1 can1-100*	Euroscarf
MLY701	Diploid issued from BM64-1A × BM64-1B cross	This work
MLY702	Isogenic to MLY701 except *ENO2/eno2Δ1::kanMX4*	This work
MLY703	Isogenic to MLY701 except *ENO2/eno2Δ2::HIS3*	This work
MLY704	Meiotic segregant of MLY702 diploid: *MAT***a** *ura3-1 trp1Δ2 leu2-3,112 his3-11 ade2-1 can1-100 eno2Δ1::kanMX4*	This work
MLY708	Meiotic segregant of MLY703 diploid: *MAT*α *ura3-1 trp1Δ2 leu2-3,112 his3-11 ade2-1 can1-100 eno2Δ2::HIS3*	This work
MLY713	Diploid issued from Y07286 × MLY708 cross	This work
MLY714	MLY713 diploid transformed with pML180 (*ScENO2*/pRS416)	This work
MLY719	Meiotic segregant of MLY714 diploid: *MAT*α *ura3-1 trp1Δ2 leu2-3,112 his3-11 ade2-1 YGR254w*[a]*::kanMX4 eno2Δ2::HIS3* + pML180 (*ScENO2*/pRS416)	This work

[a] *YGR254w, ScENO1.*

and ScEno1 of *S. cerevisiae*, respectively, and 64% identical to human α-enolase (Swiss-Prot accession P06733).

A PCR-based gene deletion cassette for *RAG17/KlENO* (Figure 1; Table 1; see MATERIALS AND METHODS for details) was used to disrupt the gene in the MW270-7B strain. Southern blot analysis confirmed the *KlENO* disruption in some G418[R] transformants, such as MWK3 (data not shown). We also demonstrated that the *KlENO*

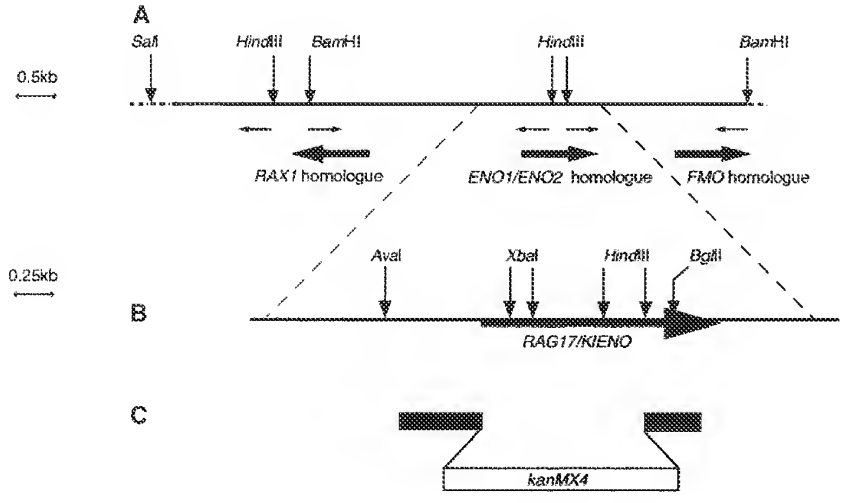

FIGURE 1.—(A) Restriction map of recombinant plasmid pMW1 and localization of the three ORFs (large arrows) identified by nucleotide sequences. Small arrows indicate the direction of DNA sequencing. (B) Restriction map of the *RAG17/KlENO* locus. (C) Disruption cassette of the *KlENO* gene with *kanMX4* marker (open box) to construct the MWK3 strain (Table 1). The solid boxes indicate genomic sequences (see MATERIALS AND METHODS).

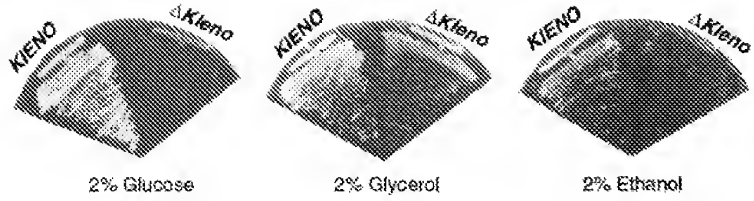

FIGURE 2.—Growth phenotype of the *Kleno* null mutant strain. The wild-type strain MW270-7B (*KIENO*), and the isogenic mutant strain MWK3 (Δ*Kleno*) were streaked onto single colonies on 2% glucose, 2% glycerol, and 2% ethanol minimal plates. The photographs were taken after 3 days of incubation at 28°.

locus is modified in the original *rag17-1* mutant (MWK1), suggesting an ectopic integration of the *rag4::URA3* gene disruption cassette in the *KIENO* gene. Like the *rag17-1* mutation, the *Kleno* null mutation leads to a Rag$^-$ phenotype. The allelism of *Kleno* with *rag17-1* was confirmed by the absence of complementation of the *Kleno* null mutation (strain MWK3) by the *rag17-1* mutation (MW352-2D) in a diploid constructed by crossing these two mutants. Thus, the cloned *KIENO* gene indeed corresponds to the *RAG17* locus.

Growth phenotype of the *Kleno* mutant: Growth of the *Kleno* null mutant is severely reduced on media containing either glucose or glycerol as the sole carbon source (Figure 2). In addition, the mutant strain is unable to grow on ethanol as carbon source. It is noteworthy that the *Kleno* mutant can grow slowly on complete glucose medium: it exhibits a 4.5-fold increase of doubling time as compared to wild-type cells (450 min *vs.* 110 min; data not shown). Better growth of mutant cells on complete medium is probably supported by other carbon sources (*e.g.*, amino acids) in this medium.

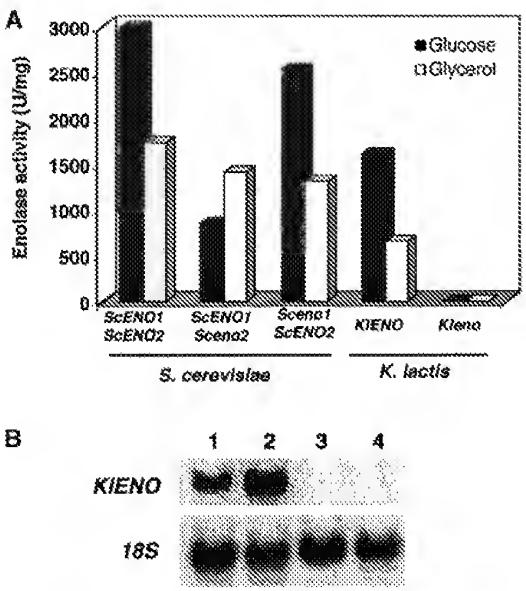

FIGURE 3.—(A) Enolase activity in wild-type and enolase mutant strains of *K. lactis* and *S. cerevisiae*. Enzyme activity was assayed as described in MATERIALS AND METHODS from cells grown on glucose or glycerol. Specific activities are expressed as micromoles of the product formed per minute and per milligram of proteins. *S. cerevisae* strains were BM61-1A (*ScENO1 ScENO2*), MLY704 (*ScENO1* Δ*Sceno2*), and Y07286 (Δ*Sceno1 ScENO2*). *K. lactis* strains were MW270-7B (*KIENO*) and MWK3 (Δ*Kleno*). (B) Northern blot analysis of *KIENO* mRNA. Each slot was loaded with ~5–10 μg of total RNA and electrophoresed on a 1.2% agarose-formaldehyde gel. The probes used are described in MATERIALS AND METHODS. Lane 1, MW270-7B (*KIENO*) strain grown on 2% glycerol; lane 2, MW270-7B (*KIENO*) strain grown on 2% glucose; lane 3, MWK3 (Δ*Kleno*) strain grown on 2% glycerol; lane 4, MWK3 (Δ*Kleno*) strain grown on 2% glucose.

Enolase activity of *Kleno* mutant and *KIENO* expression: No detectable enolase activity is present in the Δ*Kleno* mutant, regardless of the substrate used (glycerol or glucose; Figure 3A). This strongly suggests that *KIENO* is the single enolase-encoding gene in *K. lactis*. Presence of the *KIENO* sequence in a single copy and absence of other related sequences in the genome was confirmed by low-stringency Southern blotting (data not shown). In contrast, in *S. cerevisiae*, which possesses two enolase genes, enolase activity is still detectable in Δ*Sceno1* or Δ*Sceno2* single mutants grown on glucose or on glycerol. It was not possible to assay enolase activity in the double-mutant Δ*Sceno1*Δ*Sceno2* since it is inviable on glucose or glycerol media (see below).

KIENO transcription was examined by a Northern blot analysis. The level of the *KIENO* transcript is slightly higher (twofold) when the cells are grown on 2% glucose (Figure 3B) than when they are cultivated on 2% glycerol. This result is consistent with the increased level of enolase activity detected in glucose-grown cells as compared to glycerol-grown cells (Figure 3A). In the null mutant, no transcript could be detected whatever the carbon source used. These results demonstrate that *K. lactis* possesses the single gene *KIENO* coding for an enolase and expressed under glycolytic as well as neoglucogenic conditions.

The *KIENO* gene complements the Δ*Sceno1* Δ*Sceno2* mutations of *S. cerevisiae*: The nucleotide sequences of the two enolase-encoding genes of *S. cerevisiae*, *ScENO1* and *ScENO2*, are >90% identical. The major difference between the two genes is in their 5′ noncoding sequence. Therefore, they are expressed differently: the gluconeogenic gene *ScENO1* is constitutively expressed (COHEN *et al.* 1987); the glycolytic gene *ScENO2* is induced by glucose (MCALISTER and HOLLAND 1982; COHEN *et al.* 1986). The *Sceno1* null mutation has no phenotype (MCALISTER and HOLLAND 1982); the Δ*Sceno2* mutant grows more slowly on glucose than does the wild-type strain (NIEDENTHAL *et al.* 1999). Meiotic analysis of the diploid MLY713 (*ScENO2*/Δ*Sceno2::HIS3 ScENO1*

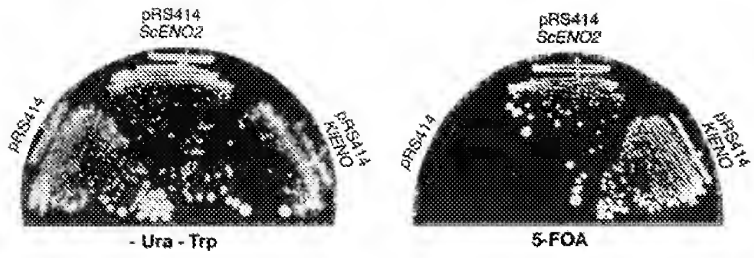

FIGURE 4.—Complementation of the Δ*Sceno1* Δ*Sceno2* double mutation of *S. cerevisiae* by the *KlENO* gene. The Δ*Sceno1*Δ*Sceno2* cells (strain MLY719, Table 1), rescued with the pML180 plasmid (*ScENO2* gene cloned in the *URA3* vector pRS416), were transformed with *TRP1* vector pRS414 (empty vector) or with the *ScENO2* and *KlENO* genes individually carried by pRS414. Transformants were grown in parallel on uracil-less, tryptophan-less, and 5-FOA medium. The plates were incubated for 3 days at 28° before the photographs were taken.

Δ*Sceno1::kanMX4*) showed that no G418^R His$^+$ spores are viable on YP medium containing either 2% glucose or 2% glycerol, but viable G418^R His$^+$ spores could be obtained on YP medium containing 0.1% glucose + 2% ethanol. Under those conditions the Δ*Sceno1*Δ*Sceno2* mutant probably obtains energy by respiration, which is derepressed at low concentrations of glucose.

To test if the *KlENO* gene can complement the growth defect of Δ*Sceno1*Δ*Sceno2*, we sporulated the MLY713 diploid (Table 1) carrying a *CEN-URA3* plasmid containing the *ScENO2* gene. A meiotic segregant of a complete tetrad, MLY719 (Ura$^+$ His$^+$ G418^R Trp$^-$), was transformed in parallel with a *CEN-TRP1* plasmid carrying either *KlENO* or *ScENO2* (Table 1). In both cases the transformants were able to grow on 5-FOA medium, which counterselects for Ura$^+$ cells (Figure 4). As a control, the same strain was transformed with the empty pRS414 plasmid and found unable to lose the plasmid carrying *ScENO2* (pML180; *i.e.*, this strain cannot grow on 5-FOA medium). We conclude that *KlENO* restores viability to the Δ*Sceno1*Δ*Sceno2* mutant of *S. cerevisiae*. Thus, *KlENO* is a functional homolog of the *ENO* genes of *S. cerevisiae*. The reciprocal heterologous complementation was confirmed: *ScENO2*, cloned in a *K. lactis* centromeric vector (pML187; see MATERIALS AND METHODS), complements the Rag$^-$ phenotype of the *Kleno* mutant (data not shown).

***KlENO* is required for expression of genes encoding glucose permeases and glycolytic genes and their regulators:** Northern blot analysis presented in Figure 5 shows that the disruption of *KlENO* results in a severe reduction of transcript levels of both glucose transporter genes, *RAG1* and *HGT1*. However, the transcription of *HGT1* was less affected than that of *RAG1*. The transcription of the hexokinase (*RAG5, KlHXK*) and pyruvate decarboxylase gene (*RAG6, KlPDC*) is also impaired in the mutant. In contrast, the phosphoglucose isomerase gene (*RAG2, KlPGI*) is not affected (Figure 5).

The reduction of the transcription of genes encoding glycolytic enzymes and glucose transporters could result from a direct effect on these genes or it could be indirect, possibly through effects on the expression of genes encoding their regulators. One of these regulators—the glucose sensor, a positive regulator of *RAG1*—is encoded by *RAG4* (BETINA *et al.* 2001). Another one, *SCK1*, codes for a helix-loop-helix type DNA-binding transcription factor, homologous to *SGC1* of *S. cerevisiae*, which is required for full expression of glycolytic genes and of the glucose carrier gene *RAG1* (LEMAIRE *et al.* 2002). *KlGCR1* and *KlGCR2* of *K. lactis* are the orthologs of the positive regulatory genes *GCR1* and *GCR2* of *S. cerevisiae* (HAW *et al.* 2001; NEIL *et al.* 2004). The *KlGCR1* and *KlGCR2* genes, like *SCK1*, appear to positively control

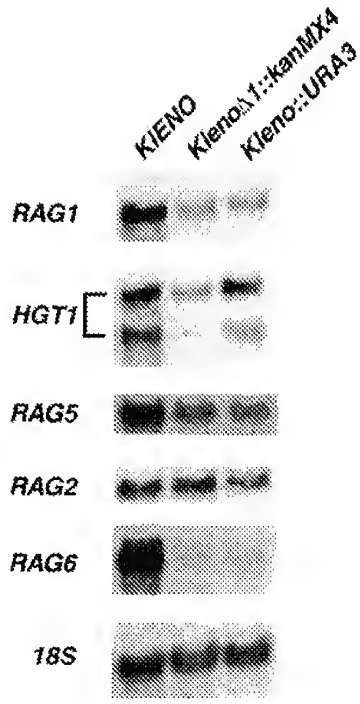

FIGURE 5.—Effect of the disruption of *KlENO* on the transcription of glucose-transporter and glycolytic genes. Approximately 5–10 μg of total RNA extracted from cells grown on 2% glucose was loaded in each slot. Strains used were MW270-7B (*KlENO*), MWK3 (*KlenoΔ1::kanMX4*), and MWK1 (carrying the original *Kleno::URA3* mutation) (Table 1). Electrophoresis conditions were as in Figure 3. The probes used to detect *RAG1, HGT1, RAG5* (*KlHXK* coding for hexokinase), *RAG2* (*KlPGI* coding for phosphoglucose isomerase), *RAG6* (*KlPDC* coding for pyruvate decarboxylase), and *18S* transcripts are described in MATERIALS AND METHODS. As already known, two mRNA are detected for *HGT1* (BILLARD *et al.* 1996). *18S* mRNA was used as an internal standard.

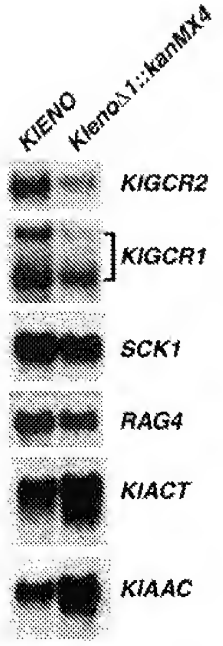

FIGURE 6.—Northern blot analysis of *KlGCR1*, *KlGCR2*, *SCK1*, and *RAG4* transcription in *Kleno* null mutant. Approximately 5 μg of poly(A$^+$) mRNA prepared from cells grown on 2% glucose was loaded in each slot. Strains used were MW270-7B (*KlENO*) and MWK3 (*KlenoΔ1::kanMX4*). The probes used are described in MATERIALS AND METHODS.

the expression of glycolytic genes and *RAG1* (NEIL *et al.* 2004). The amount of each of these transcripts was decreased compared to the wild-type strain in the enolase mutant strain (Figure 6). The transcript levels of these genes were reduced approximately five- to sevenfold in the mutant relative to the reference *KlACT* and *KlAAC* transcripts (data not shown).

A robust glycolytic flux is necessary for the full expression of *RAG1*: *KlENO* is the second glycolytic gene controlling the *RAG1* regulation to be identified. The *RAG5* gene, coding for the single hexokinase in *K. lactis*, was already known to be required for the full expression of *RAG1* (PRIOR *et al.* 1993). Enolase and hexokinase are suspected to harbor both catalytic and regulatory functions (BISSON and FRAENKEL 1983; ENTIAN and FRÖHLICH 1984; PRIOR *et al.* 1993; FEO *et al.* 2000; SUBRAMANIAN and MILLER 2000), but our data suggest a more general hypothesis: the overall glycolytic flux may regulate glucose transport.

To investigate this hypothesis, we analyzed the expression of *RAG1* in several mutants that are defective for different steps of the glycolytic or fermentation pathways (Figure 7A): Δ*Klhxk* (hexokinase), Δ*Klpgi* (phosphoglucoisomerase), Δ*Klpgk* (phosphoglycerate kinase), Δ*Kleno* (enolase), and Δ*Klpdc* (pyruvate decarboxylase). The results showed that *RAG1* expression is significantly reduced in Δ*Klhxk*, Δ*Klpgk*, and Δ*Kleno* mutants (Figure 7B) in which the glycolytic flux is blocked. However, the Δ*Klpgi* mutant, which can bypass the glycolytic block through the pentose phosphate pathway (JACOBY *et al.* 1993; GONZALEZ SISO *et al.* 1996), has little or no effect. The Δ*Klpdc* mutation that blocks the first step of fermentation following glycolysis has no impact on *RAG1* transcription. These findings suggest that glycolytic flux is required for full expression of *RAG1*.

Interestingly, regulation of glucose uptake by glycolytic flux in *S. cerevisiae* has been suggested (BISSON *et al.* 1993). We tested whether the activation of *HXT1* (most closely related to *RAG1*) expression is impaired in the enolase mutant of *S. cerevisiae* grown on glucose. No effect on expression of an *HXT1-LacZ* fusion was observed in the single *eno1* or *eno2* mutants (data not shown). This negative result is not necessarily conclusive, because both single mutants retain some enolase activity (Figure 3A). Unfortunately, this experiment cannot be performed with the double-mutant *eno1 eno2* since this mutant cannot grow on glucose (see Figure 2).

A reduced growth rate does not affect *RAG1* transcription: The glycolysis block in the *Kleno* mutant leads to a severe growth defect (Figure 2). The *Klhxk* and *Klpgk* mutants, but not the *Klpgi* and *Klpdc* mutants, show a similar growth defect (GOFFRINI *et al.* 1991; PRIOR *et al.* 1993; BIANCHI *et al.* 1996; data not shown). Thus, it remained possible that the *RAG1* transcriptional defect in the *Klhxk*, *Klpgk*, and *Kleno* mutants could be due to their reduced growth rate rather than to their reduced glycolytic capability. To investigate this possibility, a wild-type strain (MW270-7B, *KlENO*) was grown in YPG with or without different growth inhibitors. We used antimycin A and potassium cyanide (KCN), which block the respiratory chain reaction, and geneticin (G418), which inhibits protein synthesis. Figure 8A shows that these compounds inhibit the growth of *K. lactis* on YPG. However, Northern blot analysis (Figure 8B) demonstrated that the *RAG1* gene is still inducible by glucose in nondividing cells in the presence of antimycin A, KCN, or G418. This demonstrates that the *RAG1* gene is induced to similar levels in dividing and nondividing cells. These results support the idea that the defect in *RAG1* transcription in the *Kleno*, *Klhxk*, and *Klpgk* mutants is caused by reduced glycolytic flux rather than by impaired growth.

DISCUSSION

We have shown that the *KlENO* gene, which encodes enolase, the glycolytic enzyme that catalyzes the reversible conversion of 2-phosphoglycerate to phosphoenolpyruvate, is required for normal regulation of expression of *RAG1*, encoding the low-affinity glucose permease in *K. lactis*. Glycolytic enzymes have been extensively studied and characterized at the structural and biochemical level. Recently, interest in glycolytic enzymes has been revived due to their implications in other biological pathways. In *S. cerevisiae*, the hexokinase PII, encoded

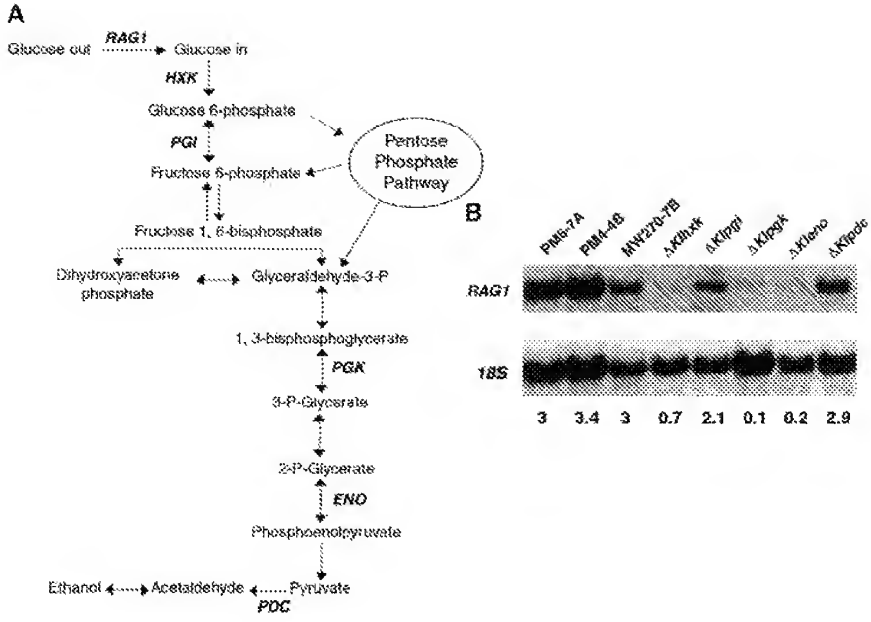

FIGURE 7.—Northern blot analysis of *RAG1* in mutant affected at different steps of glycolysis (A). Approximately 5–10 μg of total RNA extracted from cells grown on 2% glucose was loaded in each slot. Electrophoresis conditions were as in Figure 3. The probes used to detect *RAG1* and *18S* transcripts are described in MATERIALS AND METHODS. (B) The strains MW270-7B, PM4-4B, and PM6-7A are the isogenic wild-type strains of the different mutants used. Δ*Klhxk*, MWK11 strain; Δ*Klpgi*, MWK12 strain; Δ*Klpgk*, MWK45 strain; Δ*Kleno*, MWK3 strain; Δ*Klpdc*, MWK13 strain. These strains are described in Table 1. *18S* mRNA was used as an internal standard. The hybridization signals have been quantified with a phosphoimager (MATERIALS AND METHODS) and numbers below the panel indicate the ratio of *RAG1:18S*.

by the *HXK2* gene, is involved in glucose repression (RANDEZ-GIL *et al.* 1998). In fact, nuclear localization of Hxk2 appears to be glucose regulated, and it interacts *in vivo* with Mig1, the transcriptional repressor of many glucose-repressed genes (AHUATZI *et al.* 2004). In addition, in *K. lactis* and *S. cerevisiae*, hexokinase is also required for full glucose induction of *RAG1* and *HXT* gene expression (PRIOR *et al.* 1993; ÖZCAN and JOHNSTON 1999). Several pieces of evidence suggest that enolase is a multifunctional protein playing a crucial role in transcriptional and physiological processes (reviewed in PANCHOLI 2001). For example, enolase has been identified as a heat-shock protein in *S. cerevisiae* (IIDA and YAHARA 1985) and has been found to have transcriptional regulatory functions in larger eukaryotes. In the latter case, these functions occur through binding of enolase to certain gene promoters: *c-myc* (FEO *et al.* 2000; SUBRAMANIAN and MILLER 2000) or *STZ/ZAT10*, encoding a transcriptional repressor in Arabidopsis (LEE *et al.* 2002).

The finding that the expression of *RAG1* as well as several genes encoding glycolytic enzymes is affected in the Δ*Kleno* mutant suggests that enolase could play a regulatory role in *K. lactis* in addition to its catalytic activity. However, we have not yet demonstrated a direct role for enolase in the transcriptional regulation of *RAG1*. In addition to enolase it was already known that induction of *RAG1* expression by glucose is dependent on hexokinase activity (PRIOR *et al.* 1993). Since the loss of any one of the glycolytic steps tested, except that of phosphoglucoseisomerase, severely reduces *RAG1* transcription, we believe that glucose metabolism generates a signal that induces *RAG1* expression. We cannot, however, exclude the possibility that enolase has a general regulatory function on other genes.

The defect in *RAG1* transcription in mutants blocked in glycolysis suggests the existence of regulatory mechanisms that prevent expression of genes encoding glucose transporters if a functional glycolytic pathway cannot be maintained. MILEOWSKI *et al.* (2001) previously showed that the absence of glucose transporters (hence glucose uptake) impaired the induction of *KHT1/RAG1* expression by high levels of glucose. Altogether, these data suggest that an intracellular glucose-sensing mechanism relying on glucose metabolism through glycolysis may ensure optimal glucose uptake by activating expression of the gene encoding the low-affinity glucose transporter. This intracellular pathway presumably collaborates with the extracellular glucose-sensing mechanism operating through the Rag4 glucose sensor in the cell membrane (BETINA *et al.* 2001). In *E. coli*, the expression of *pts*G, encoding the major glucose transporter $IICB^{Glc}$, also requires glycolytic flux (KIMATA *et al.* 2001). Whatever the mechanism, regulation of glucose uptake by glycolytic flux seems to have been conserved from bacteria to yeasts.

We have a few clues to the mechanism by which glycolytic flux regulates expression of *RAG1* and glycolytic genes. The inactivation of *KlENO* leads to a severe reduction in expression of the regulatory genes *KlGCR1*, *KlGCR2*, *SCK1*, and *RAG4* (Figure 6). Thus, the effects of enolase on expression of genes encoding glycolytic enzymes and glucose transporters may be indirect. *KlGCR1*, *KlGCR2*, and *SCK1* are required for the full expression of glycolytic genes (LEMAIRE *et al.* 2002; NEIL *et al.* 2004). The KlGcr1/KlGcr2 complex directly regulates glycolytic gene expression through binding of KlGcr1 to glycolytic gene promoters (NEIL *et al.* 2004). An interaction between Sck1 and *K. lactis* glycolytic promoters is also probable since its *S. cerevisiae* ortholog

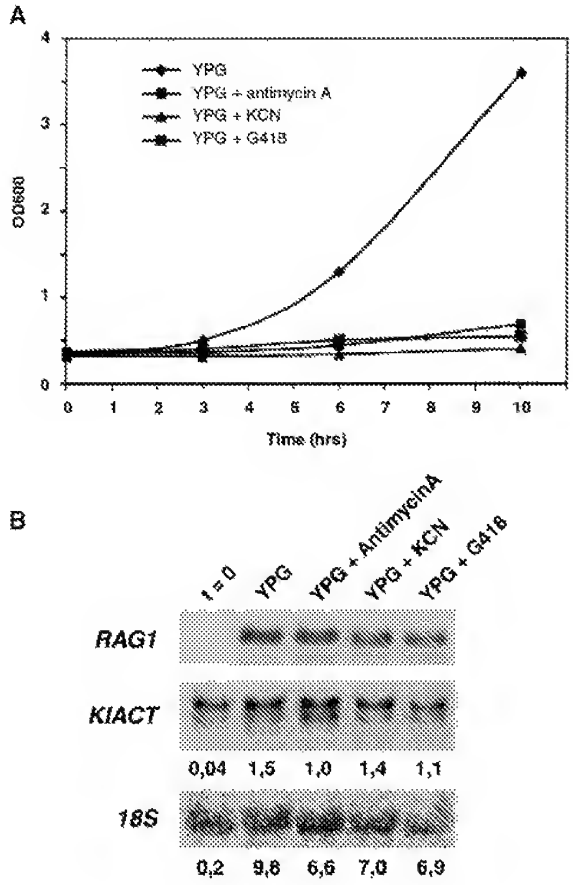

FIGURE 8.—Effect of the growth rate on *RAG1* transcription. (A) Inhibition of *K. lactis* growth by antimycin A, KCN, or G418. The MW270-7B (*KlENO*) strain was first grown in 50 ml YP medium containing 2% glycerol to an OD_{600} of 2. Cells were harvested, washed, and resuspended in sterile cold water and aliquots (~15 OD_{600}) were diluted in 50 ml YPG (2% glucose) or YPG containing 5 μM antimycin A, 5 mM KCN, or 100 μg/μl G418. Cultures were kept agitated at 28° during 10 hr and OD_{600} was checked at 0, 3, 6, and 10 hr. (B) Northern blot analysis of *RAG1*, *KlACT*, and *18S* transcript levels. Total RNA was extracted from the YPG culture at $t = 0$ (shift on glucose) and from the four cultures at $t = 10$ hr. Electrophoresis conditions and quantification were done as described in the Figure 6 legend. The probes used to detect *RAG1*, *KlACT*, and *18S* transcripts are described in MATERIALS AND METHODS. *KlACT* and *18S* mRNA was used as internal standards and numbers below the corresponding panels indicate the ratio of *RAG1*:*KlACT* or *RAG1*:*18S*.

(Sgc1) binds to the *ENO1* promoter (SATO *et al.* 1999). Hence, the transcriptional defect of *RAG5*/*RAG6* genes in the *Kleno* mutant is almost certainly the consequence of the low level of expression of *KlGCR1*, *KlGCR2*, and *SCK1*. Interestingly, these transcription factors are also required for the full glucose induction of *RAG1* expression (LEMAIRE *et al.* 2002; NEIL *et al.* 2004). At least in the case of KlGcr1/KlGcr2, this effect seems indirect since KlGcr1 does not bind the *RAG1* promoter *in vitro* (NEIL *et al.* 2004) and thus may be a consequence of the reduced glycolytic flux in the *Klgcr1*/*Klgcr2* mutants. Although we cannot exclude a cumulative effect of the reduced expression of *KlGCR1*, *KlGCR2*, *SCK1*, and *RAG4* on *RAG1* expression, we favor the hypothesis that a product of glycolysis may control the activity of an unidentified regulator. Such metabolic controls have been described already in *S. cerevisiae*. For instance, the Mcm1 transcriptional regulator is regulated post-transcriptionally by the glycolytic flux (CHEN and TYE 1995). Moreover, the efficient transcription of yeast AMP biosynthetic genes requires interaction between the transcription factors Bas1p and Bas2p, and this interaction is promoted in the presence of a metabolic intermediate (SAICAR) of this biosynthetic process (REBORA *et al.* 2001).

On the basis of the present findings, we propose that the regulation of *RAG1* expression by glucose involves two pathways: (i) a pathway involving the glucose sensor Rag4 that responds to extracellular glucose availability (BETINA *et al.* 2001) and (ii) a pathway responding to an intracellular signal generated by glycolysis. Together, these pathways can be considered to be an autoregulatory device for the fermentative utilization of sugars in yeast. The *K. lactis* system with its nonredundancy of the genes of glucose metabolism, the clear Rag^- phenotype associated with glycolytic mutations, and its metabolic properties appears to be a suitable tool to study intracellular glucose sensing in yeast.

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Impact of Mitochondrial Function on Yeast Susceptibility to Antifungal Compounds

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ABSTRACT. *Saccharomyces cerevisiae pel1* and *crd1* mutants deficient in the biosynthesis of mitochondrial phosphatidylglycerol (PG) and cardiolipin (CL) as well as *Kluyveromyces lactis* mutants impaired in the respiratory chain function (RCF) containing dysfunctional mitochondria show altered sensitivity to metabolic inhibitors. The *S. cerevisiae pel1* mutant displayed increased sensitivity to cycloheximide, chloramphenicol, oligomycin and the cell-wall perturbing agents caffeine, caspofungin and hygromycin. On the other hand, the *pel1* mutant was less sensitive to fluconazole, similarly as the *K. lactis* mutants impaired in the function of mitochondrial cytochromes. Mitochondrial dysfunction resulting either from the absence of PG and CL or impairment of the RCF presumably renders the cells more resistant to fluconazole. The increased tolerance of *K. lactis* respiratory chain mutants to amphotericin B, caffeine and hygromycin is probably related to a modification of the cell wall.

Abbreviations

CL	cardiolipin			PG	phosphatidylglycerol
mtDNA	mitochondrial DNA			PGPS	phosphatidylglycerophosphate synthase
MIC	minimum inhibitory concentration			RCF	respiratory chain function
Amb	amphotericin B	Cyh	cycloheximide	Ket	ketoconazole
Bif	bifonazole	Eco	econazole	Mic	miconazole
Caf	caffeine	Flu	fluconazole	Muc	mucidin
Clm	chloramphenicol	Hyg	hygromycin B	4-NQO	4-nitroquinoline *N*-oxide
Clo	clotrimazole	Itr	itraconazole	Oli	oligomycin
Cpf	caspofungin				

Mitochondria play a fundamental role in eukaryotic cell physiology producing cellular energy and other essential metabolites. By integrating numerous death signals mitochondria are also involved in the control of apoptosis. The biogenesis of functional mitochondria depends on the coordinated expression of two genomes, nuclear and mitochondrial, a cross-talk essential for cell life and death. While mitochondria contain their own DNA (mtDNA) encoding a handful of proteins, the vast majority of mitochondrial proteins are synthesized on cytosolic ribosomes and imported post-translationally into the organelle.

Mitochondrial genome integrity is essential for the viability of most yeast species. Loss of mtDNA or mitochondrial protein synthesis is lethal for *petite*-negative yeast species, *e.g.*, *Kluyveromyces lactis*. The *K. lactis* cells are not able to tolerate the absence of both electron–proton transport pumping and ATP synthesis components of oxidative phosphorylation (Clark-Walker and Chen 2001). However, such a profound change can be tolerated by *petite*-positive *Saccharomyces cerevisiae*, readily forming respiratory-deficient *petite* mutants that lost mtDNA after treatment with DNA targeting drugs (Bulder 1964). On the other hand, mutations in several nuclear genes transform *S. cerevisiae* into a *petite*-negative species which no longer tolerates the cytoplasmic "*petite*" state: *op1/aac2*, deficient in ATP/ADP translocase (Kolarov *et al.* 1990), *pel1/pgs1* deficient in PGPS (Janitor *et al.* 1996; Chang *et al.* 1998). The *pel1* (*petite* lethal) mutation, initially described as a nuclear mutation displaying synthetic lethal phenotype with mitochondrial *rho* mutations in *S. cerevisiae* (Šubík 1974), was found to be associated with CL deficiency (Janitor *et al.* 1996). It has been demonstrated that *PEL1* (renamed *PGS1*) encodes PGPS, the 1st enzyme in the CL biosynthesis pathway (Chang *et al.* 1998). The Pel1 and/or Pgs1p is localized to mitochondria, where both PG and CL are synthesized (Džugasová *et al.* 1998; Daum *et al.* 1998; Schlame *et al.* 2000). Disruption of *PEL1* (*PGS1*) results in complete loss of both PG and CL. CL (a phospholipid found predominantly in the inner mitochon-

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drial membrane) is essential for the biogenesis and proper RCF (Jiang *et al.* 2000; Vreken *et al.* 2000; Pfeiffer *et al.* 2003), ATP synthase (Koshkin and Greenberg 2002) and of ATP/ADP translocase (Hoffmann *et al.* 1994). PG can substitute for some essential functions of CL, both PG and CL being required for maintaining mtDNA (Džugasová and Šubík 2005; Zhong *et al.* 2005).

Due to the connection between the functional state of mitochondria and the activation of pleiotropic drug resistance network in *S. cerevisiae* (Hallstrom and Moye-Rowley 2000; Devaux *et al.* 2002), our aim was to analyze the possible influence of mitochondrial dysfunction resulting from altered mitochondrial membrane lipid composition in *petite*-positive *S. cerevisiae* as well as impaired RCF in *petite*-negative *K. lactis* on the cell's sensitivity to metabolic inhibitors and antifungal compounds.

MATERIAL AND METHODS

Media, culture conditions and strains. For the yeast strains used *see* Table I. Cells were grown at 30 °C in YPD complex medium with glucose (in %: glucose 2, bacteriological peptone 2, yeast extract 1), in YPG complex glycerol medium (in %: glycerol 2, bacteriological peptone 2, yeast extract 1) or YNB minimal medium (*Difco*) containing 0.67 % Yeast Nitrogen Base without amino acids, 2 % glucose, and supplemented with the auxotrophic requirements (40 mg/L). Solid media were prepared in 2 % *Difco* agar (all percentages are *M/V*). Respiration-deficient *petite* mutants from *S. cerevisiae crd1* strain were prepared by ethidium bromide (25 μmol/L) mutagenesis (Slonimski *et al.* 1968).

Table I. Yeast strains used

Strain	Genotype	Reference
Saccharomyces cerevisiae		
DW4-2A/YEp352/*PEL1*	MATa *ade2-1 ura3-1 trp1-1 his3-11 pel1*/YEp352/*PEL1*	Jamtor and Šubík 1993
DW4-2A/YEp352 (*pel1*)	MATa *ade2-1 ura3-1 trp1-1 his3-11 pel1*/YEp352	*this study*
FY1679 (*CRD1*)	MATa *ura3-52 trp1Δ63 his3 Δ200 CRD1*	Tuller *et al.* 1998
FYC/5 (Δ*crd1*)	MATa *ura3-52 leu2 trp1Δ63 his3 Δ200 crd1*::kanMX4	*ditto*
FYC/5 (Δ*crd1*/*rho*$^-$)	MATa *ura3-52 trp1Δ63 his3 D200 crd1*::kanMX4/*rho*^{0}	*this study*
Kluyveromyces lactis		
JBD100	a *trp1 lac4-1 ura3-100* Rag$^+$	Heus *et al.* 1990
JBD100/M1	a *trp1 lac4-1 ura3-100 gly1* Rag$^+$	Gbelská *et al.* 1996
JBD100/M5	a *trp1 lac4-1 ura3-100 gly2-2* Rag$^+$	*ditto*
JBD100/M7	a *trp1 lac4-1 ura3-100 gly3-2* Rag$^+$	*ditto*
PM6-7A	a *ura41 ade2* Rag$^+$	Chen *et al.* 1992
PM6-7A/Δ*cox18*	a *ura41 ade2KI cox18*::URA3	Hikkel *et al.* 1997

Drug susceptibility was tested qualitatively by spot assay. The strains were grown overnight at 30 °C in liquid YNB medium, cells were diluted to a concentration of 10/nL (*i.e.* 10^7 cells per mL) in sterile water and 10 mL of the cell suspension and 10-fold serial dilutions of cells were spotted onto complex and/or minimal plates with glucose or glycerol supplemented with various concentrations of the drugs tested, followed by incubation (3–5 d, 30 °C). Susceptibility to metabolic inhibitors, *i.e.* Cyh (*Sigma*) dissolved in ethanol, 4-NQO (*Sigma*), dissolved in acetone, Hyg (*Boehringer Mannheim*), Caf (*Fluka*), dissolved in Me_2SO, and antifungal compounds Cpf, Flu (*Pfizer Amboise*, France), dissolved in sterile water, Ket (*Janssen Pharmaceuticals*, Belgium), Mic (*Sigma*), Clo (*Sigma*), Bif (*Sigma*) dissolved in Me_2SO, was tested on YNB medium with glucose excepting Oli (*Sigma*) and Muc (*Sigma*) dissolved in ethanol, and Clm (*Sigma*) dissolved in Me_2SO, that were tested on YPG. Sensitivity was also assessed using zone-inhibition assays. Approximately 10^7 stationary-phase cells were plated onto minimal glucose agar. Filter discs (∅ 6 mm) soaked with an appropriate amount of antifungal compounds were placed on the plates, which were then incubated at 30 °C for 3–5 d before determining the diameter of the zone of growth inhibition.

Plasmid and transformation procedure. Cells of the strain DW4-2A were transformed using the empty high-copy episomal plasmid YEp352 (2μm, *URA3*, amp^R) or plasmid YEp352/*PEL1* containing the standard allele of the *PEL1* gene (Džugasová *et al.* 1998) using the lithium acetate procedure (Sambrook *et al.* 1989).

RESULTS AND DISCUSSION

S. cerevisiae *mutants deficient in PG and CL are sensitive to metabolic inhibitors.* Sensitivity to a broad variety of metabolic inhibitors correlates with mitochondrial function (Zhang and Moye-Rowley 2001; Devaux *et al.* 2002). CL and PG (anionic phospholipids) are synthesized inside the mitochondria, essential for the proper function of the organelle. Therefore the sensitivity of *pel1* mutant (deficient in PG and CL), *crd1* mutant (deficient in CL) and *crd1/rho* mutant (deficient in CL and mtDNA) to several metabolic inhibitors (Cyh, Clm, 4-NQO, Oli, Muc) was compared with that of the corresponding wild-type strains *pel1*/YEp352 – *PEL1* and *CRD1*, respectively. The simultaneous absence of PG and CL in the *pel1* mutant renders the cells more sensitive to Cyh, Oli and Clm in comparison with the corresponding wild-type strain (Table II). The MIC of Muc was similar for both mutants (*pel1*, *crd1*) and the corresponding wild-type

Table II. Sensitivity of *S. cerevisiae* strains to metabolic inhibitors (MIC, μg/mL)

Strain	Cyh[a]	4-NQO[b]	Oli[c]	Clm[d]	Muc[e]
DW4-2A/YEp352/*PEL1*	0.15	0.3	>1	>14	<0.025
DW4-2A/YEp352 (*pel1*)	0.05	0.2	0.6	12	<0.025
FY1679 (*CRD1*)	0.1	0.2	>0.9	14	0.05
FYC/5 (Δ*crd1*)	0.1	0.1	0.9	12	<0.025
FYC/5 (Δ*crd1*/*rho*$^-$)	0.1	0.2	–	–	–

[a]Cycloheximide 0, 0.05, 0.1, 0.15, 0.2, 0.25, 0.3–0.7
[b]4-Nitroquinoline *N*-oxide 0.1, 0.2, 0.3, 0.4–0.7
[c]Oligomycin 0.1–0.9
[d]Chloramphenicol 5–14
[e]Mucidin 0.025, 0.05, 0.075, 0.1

strains. On the other hand, the deficiency of CL alone in the *crd1* mutant or the simultaneous absence of CL and mtDNA in the double *crd1/rho* mutant did not influence the sensitivity of cells to the metabolic inhibitors. The observed effect in the *pel1* mutant may be associated with an increased drug efflux induced by dysfunctional mitochondria. The loss of mitochondrial function led to the activation of the *PDR3* gene-mediated overexpression of *PDR5* encoding a multidrug resistance efflux pump (Zhang and Moye-Rowley 2001; Devaux *et al.* 2002).

However, the presence of PG and CL is essential for physiology (Janitor and Šubík 1993; Chang *et al.* 1998; Džugasová *et al.* 1998; Gohil *et al.* 2004) and biogenesis of mitochondria (Jaing *et al.* 2000; Schlame *et al.* 2000; Ostrander *et al.* 2001; Koshkin and Greenberg 2002) and their protective role in the eukaryotic cell response to oxidative stress was also proposed (Mileykovskaya *et al.* 2005).

Mitochondrial dysfunction affects the susceptibility of petite-*positive and* petite-*negative yeasts to antifungal compounds.* The deficiency in PG and CL rendered the *S. cerevisiae pel1* mutant cells more sensitive to Ket, Mic, Bif and Amb (Table III) in comparison with the isogenic wild-type strain. Moderate increase in sensitivity to azole antifungals was also seen in the *S. cerevisiae* Δ*crd1* mutant deficient in CL only in comparison with its parental wild-type strain (*CRD1*). However, the loss of mtDNA in the Δ*crd1* mutant caused an increased sensitivity to the antifungal compounds. The *pel1* mutant deficient in PG and CL was less sensitive to Flu than its isogenic transformant containing the *PEL1* gene on a multicopy plasmid (Table III, Fig. 1). The presence of PG in the *S. cerevisiae* Δ*crd1* mutant could substitute the essential functions of CL in mitochondria and the cells behave more or less like wild-type ones. The absence of both PG and CL in the *pel1* mutant leads to mitochondrial dysfunction that is associated with the observed Flu resistance.

The sensitivity of K. lactis *mitochondrial mutants to antifungal compounds depends on RCF.* The *K. lactis* nuclear mutants deficient in cytochrome *c*, cytochrome c_1 and cytochrome oxidase were less sensitive to Flu, Mic, Clo, Ket, Bif, and Eco in comparison with the corresponding wild-type strain (Fig. 2). The results of disk diffusion assays correlated with those observed in the qualitative testing of cell sensitivity by spotting serial dilutions of cells onto YPD or minimal agar plates containing different concentrations of anti-

Table III. Sensitivity of *S. cerevisiae* strains to antifungal compounds and metabolic inhibitors (MIC)

Strain	Flu[a] μg/mL	Ket[b] μg/mL	Mic[c] μg/mL	Clo[d] μg/mL	Bif[e] μg/mL	Amb[f] μg/mL	Hyg[g] mg/mL	Caf[h] mg/mL	Cpf[i] mg/mL
DW4-2A/YEp352/*PEL1*	<14	7	0.1	0.6	5	7.5	0.1	>1	>0.75
DW4-2A/YEp352 (*pel1*)	>15	6	<0.1	0.5	2.5	5	0.1	<1	0.5
FY1679 (*CRD1*)	>15	6	0.1	0.6	>5	5	0.4	2	0.7
FYC/5 (Δ*crd1*)	12	2	<0.1	0.6	>5	5	0.4	2	0.5
FYC/5 (Δ*crd1*/*rho*$^-$)	12	<2	<0.1	0.4	2.5	2.5	0.2	<1	0.25

[a]Fluconazole 0, 10–15 μg/mL
[b]Ketoconazole 1–10 μg/mL
[c]Miconazole 0.1–0.8 μg/mL
[d]Clotrimazole 0.1–0.8 μg/mL
[e]Bifonazole 2.5, 5, 7.5, 10 μg/mL
[f]Amphotericin B 0, 2.5, 5, 7.5, 10 μg/mL
[g]Hygromycin 0.1–1 mg/mL
[h]Caffeine 0, 0.5, 1, 1.5, 2, 2.5, 3 mg/mL
[i]Caspofungin 0, 0.1, 0.25, 0.5, 0.75 mg/mL

fungal compounds. All of the *K. lactis* mutants affected in the RCF were able to grow on plates containing Flu at 15 μg/mL in contrast with the isogenic wild-type strains (Fig. 1). Taken together, we found that the loss of mitochondrial function resulting from the absence of PG and CL in the *S. cerevisiae pel1* mutant or impaired RCF in *K. lactis* mutants is linked to the increased tolerance of yeast cells to Flu.

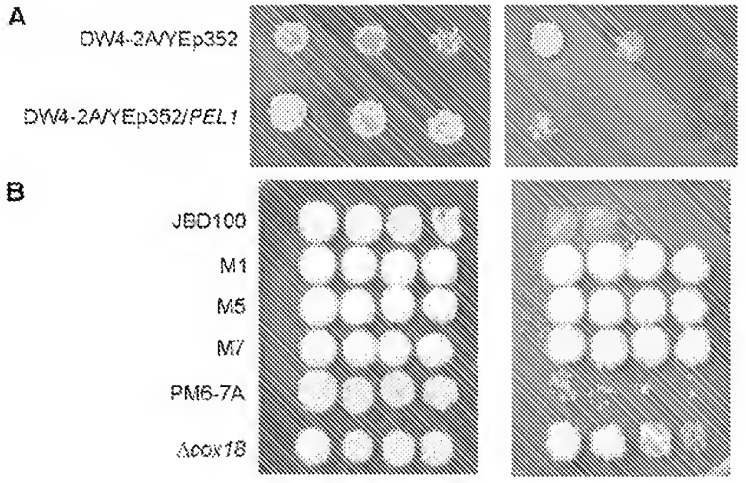

Fig. 1. Fluconazole sensitivity profiles of *S. cerevisiae* (**A**) and *K. lactis* (**B**) mutants. Wild type and mutant strains were grown in YNB medium for overnight at 30 °C, the A_{600} was normalized and 10 μL of 10-fold serial dilutions were spotted onto YNB plates with or without fluconazole, photographed after 5 d of growth at 30 °C.

Flu acts as ergosterol-depleting agent in *S. cerevisiae* and mitochondria could function as important physiological partners in the accumulation of toxic sterol intermediates in the presence of azoles (Kontoyiannis 2000). Dysfunctional mitochondria could not assist in the accumulation of toxic sterol intermediates and the cells can tolerate the presence of Flu.

Mitochondrial functions are linked to cell-wall biogenesis. Zhong *et al.* (2005) indicated the role of mitochondrial lipids PG and CL in mitochondrial functions required for cell-wall biogenesis. Therefore the sensitivity of *S. cerevisiae* mutants deficient in PG and CL to Caf, Cpf and Hyg were assessed. The absence of PG and CL in *S. cerevisiae pel1* mutant renders the cells more sensitive to Caf and Cpf in comparison with the isogenic wild-type strain (Table III). The sensitivity of *S. cerevisiae* Δ*crd1* mutant and its corresponding wild-type strain to the inhibitory compounds used were similar. The presence of PG can apparently substitute for the CL functions in the Δ*crd1* mutant. The loss of mtDNA in the Δ*crd1* mutant caused even higher sensitivity of cells to Caf, Hyg and Cpf. The results correlate with the proposed role of PG and CL in functions that are essential for cell wall biogenesis. On the other hand, the *K. lactis* mutants affected in the RCF (deficient in cytochrome *c*, cytochrome c_1 or cytochrome oxidase) showed an increased tolerance to

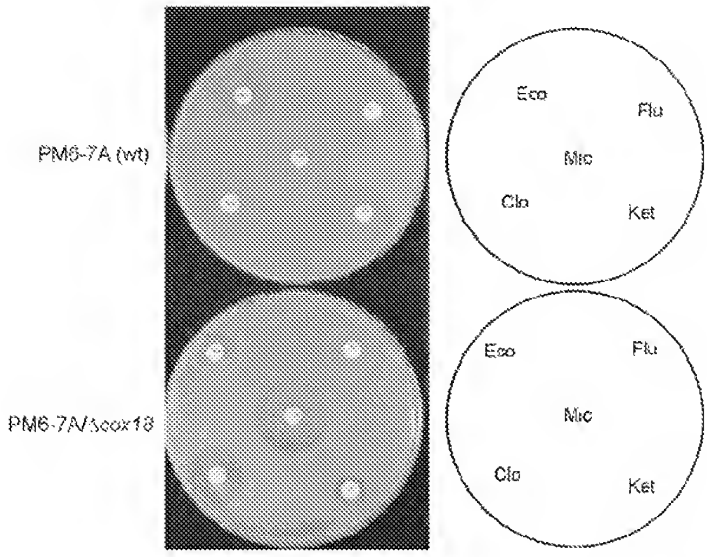

Fig. 2. Antifungal sensitivity of wild-type *K. lactis* PM6-7A and PM6-7A/Δ*cox18* mutant cells; performed by the disc diffusion assay on YNB agar plates; Eco – econazole, Flu – fluconazole, Mic – miconazole, Clo – clotrimazole, Ket – ketoconazole.

Amb, Caf and Hyg (Table IV) suggesting changes in the cell wall structure as a result of mitochondrial dysfunction. The correlation between mutations in genes related to mitochondrial functioning and yeast cell wall characteristics was first mentioned in 1980 (Evans *et al.* 1980). Later on the connection between Amb

Table IV. Sensitivity of *K. lactis* strains to antifungal compounds and metabolic inhibitors (MIC)

Strain	Flu[a] µg/mL	Bif[b] µg/mL	It[c] µg/mL	Amb[d] µg/mL	Hyg[e] µg/mL	Caf[f] mg/mL
JBD100 (wt)	20	3	7.5	0.5	25	1
JBD100/M1	>50	>10	>50	>2	50	3
JBD100/M5	>50	>10	>50	>2	50	3
JBD100/M7	>50	>10	>50	>2	50	3
PM6-7A (wt)	20	4	15	1	50	2
PM6-7A/Δ*cox18*	50	6	>15	>2	50	3

[a]Fluconazole 10, 20, 25, 30–50 µg/mL
[b]Bifonazole 0.5, 1, 1.5, 2–6, 10 µg/mL
[c]Itraconazole 2.5, 5, 7.5, 10, 15, 20, 25, 30, 50 µg/mL
[d]Amphotericin B 0, 0.5, 1, 2 µg/mL
[e]Hygromycin 5, 10, 25 , 50 µg/mL
[f]Caffeine 0, 1–4 mg/mL

resistance and modification of the cell wall in *S. cerevisiae* (Lussier *et al.* 1997; Wauters *et al.* 2001; Zhang *et al.* 2003; Agarwal *et al.* 2003; Hapala *et al.* 2005) and also in *K. lactis* has been reported (Ramanandraibe *et al.* 1998). The results obtained using both *petite*-positive and *petite*-negative species show that the functional state of yeast mitochondria influences the surface characteristics of the cell as well as the susceptibility of cells to antifungal compounds.

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